THE EFFECT OF FLUID, ELECTROLYTE AND FOOD INTAKE ON THORACIC DUCT LYMPH FLOW IN UNANAESTHETIZED RATS

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It is well known, from experiments on anaesthetized animals, that the flow of thoracic duct lymph is greater when food and fluid have recently been consumed than it is after fasting, but the possible causes of the increased flow have not been systematically studied. Anaesthesia may interfere with intestinal motility, secretion and absorption. It was, therefore, decided to conduct experiments in unanaesthetized rats, with indwelling plastic cannulae in the cisterna chyli. This paper deals mainly with the effect of fluid and electrolytes, together with changes in the flow and protein concentration of lymph after protein, carbohydrate or fat were given in fairly large volumes of fluid.

METHODS.

Male rats of an albino Wistar strain were used in all experiments. The weights varied from 180 to 270 gm., usually between 220 and 260 gm.

Operative preparation. Under ether anaesthesia, the abdominal thoracic duct was cannulated by the method of Bollman, Cain and Grindlay (1948). Polyethylene tubes of 1 mm. external diameter were used in the earlier experiments, but later transflex tubing, 1½ mm. in external diameter, was available and proved more satisfactory. A rubber-tipped plastic tube was inserted into the cardiac region of the stomach, through an incision high up on the anterior aspect, and anchored with a purse string suture. The abdominal wound was sutured and the animal placed in a restraining cage of the type devised by Bollman (1948). The use of a gastrostomy tube allowed more accurate timing and measurement of the dose of fluid or food, and obviated the possible effect on lymph flow of increased movement associated with free feeding. Lymph was continuously collected so long as the flow remained unobstructed and the animal was in good clinical condition. This period varied from 1 to 4 days in different animals.

Maintenance of hydration and lymph flow. During the first 5 to 6 hours after operation, 5 ml. of 0·9 p.c. NaCl solution was given slowly by way of the gastrostomy tube, to assist recovery from the operation and to ensure a free post-operative flow of lymph. After this, 0·3 p.c. NaCl solution was usually given overnight by the same route, at a steady rate of about 0·6 ml./hr. This was sufficient to maintain approximate fluid balance and an average lymph flow of more than 0·5 ml./hr. in the first 16 hours after operation. In early experiments, where large volumes of 0·9 p.c. NaCl solution were given in the first 16 hours after operation,

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the animals showed signs of post-operative salt intolerance (Coller, Campbell, Vaughan, Job and Moyer, 1944)—falling urinary output and progressive fluid retention. When single doses of food and fluid were given, the output of lymph and urine during the 10 to 12-hour experimental session exceeded the fluid administered by about 5 ml. At the end of the session, 5 ml. of 0·9 p.c. NaCl solution was given slowly and then Ringer-Locke or saline by automatic feeder at about 0·6 ml./hr., to maintain hydration and lymph flow overnight.

Urine was collected by means of a funnel and the volume measured, the fluid balance being assessed approximately by the progressive values of input minus urine and lymph volumes. Daily output usually somewhat exceeded intake when the animal was not allowed to correct its fluid balance by *ad lib.* fluids. The possible effect of hydration was allowed for in the design in such experiments, e.g., by repeating experiments on different post-operative days or after rest periods with variable or *ad lib.* fluid intake.

Collection of lymph. Lymph was collected in flasks and the volume measured in graduated cylinders or collected in successive short periods in graduated centrifuge tubes, dry heparin being used to prevent clotting. Clots in the plastic tubing were removed by a fine stilette with a hooked end. This was seldom necessary after the first 16 hours following operation, when transflex was used. If there was any doubt concerning an unobstructed flow of lymph the results of the experiment were discarded.

Experimental procedure. Lymph was continuously collected and fluid, with or without various foodstuffs, given by continuous drip or in single doses.

(i) *Continuous administration of fluid and electrolyte.* These experiments began at 9 a.m. on the morning following operation. Distilled water or 0·9 p.c. NaCl solution was then given for 24 hours, from 9 a.m. to 5 p.m. at 4 ml./hr., and from 5 p.m. to 9 a.m. on the next morning at about 0·7 ml./hr. The fluid was changed alternately to saline or water at the end of each 24-hour period. The rats had access to dry food (rat nuts).

(ii) *Single doses of fluid, with or without foodstuffs.* These rats had not been used in the experiments described above. The first of a series of experimental sessions began, as before, at 9 a.m. on the morning following operation. Each session consisted of three periods. In the first period, from 9 a.m. to 11 a.m. or later, no fluid or food was given. When a steady rate of flow had been maintained for not less than one hour, one of the substances to be tested was given as described below. This marked the beginning of the second period, which lasted for 3 to 4 hours. During this time the lymph flow was measured at quarter-hourly intervals for the first 1 to 2 hours after administering fluid and food, then at half-hourly intervals. The second period ended when the lymph flow had returned to the rate established in period 1, and remained steady for one hour. Period 3 followed immediately, consisting of injection of a different substance and measurement of lymph flow as before. Thus the experimental session lasted usually from 9 a.m. until 7 to 9 p.m. on each day. Between sessions, i.e., overnight, fluid only was given, as previously described, to maintain hydration and lymph flow. Substances were tested in pairs, e.g., full cream and skim milk, the order being reversed each time the experiment was repeated. Protein concentrations were estimated in the lymph collected during the initial period and, after each injection, during the first and second hours and the final period of 1 to 2 hours. The effect of oleic acid or olive oil was tested on different days, lymph being collected for 6 hours or more after injection. The rats received no food other than the small amounts injected experimentally. Each rat was submitted to two, or occasionally three, experimental sessions with different materials.

Substances administered. When a single dose was given by intra-gastric tube the volume of fluid was always 5 ml. and the time for complete injection was approximately 10 minutes, so that the purely mechanical effects of injection would be reasonably uniform. The substances injected were as follows: *Protein.* Thirty p.c. bovine serum albumin solution (Armour) diluted with distilled water to 10 gm. albumin/100 ml. *Carbohydrate.* Ten gm.

glucose (B.P.)/100 ml. water. *Fat.* 0·5 ml. olive oil (B.P.) or oleic acid, redistilled (B.P.), washed into the stomach with 4·5 ml. distilled water. *Skim milk.* Ten gm. powdered skim milk (fat 1 p.c., protein 36 p.c., carbohydrate 52 p.c.) per 100 ml. distilled water, to which 2 ml. 25 p.c. sodium citrate solution/100 ml. had been added. *Full cream milk.* A solution of full cream milk powder (fat 26 p.c. of dry weight, protein 28 p.c., carbohydrate 38 p.c.) made up in the same way as for skim milk. *Suspended rat nuts.* Rat nuts (fat 2·4 p.c., protein 21 p.c., carbohydrate 55 p.c.) were ground up to a fine powder and made up in 10 p.c. suspension (W/V) in distilled water. The dose of mixed foodstuffs or isolated components was therefore 0·5 gm. in all cases.

Analytical. Total nitrogen and N.P.N. concentrations in lymph and plasma were determined by micro-Kjeldahl digestion and direct Nesslerisation, using a Spekker photoelectric absorptiometer, N.P.N. being determined after precipitation of protein with trichloracetic acid.

Expression of results. In most instances, where mean values are given they are followed by ± standard error of mean.

RESULTS.

Effect of fluid and electrolyte intake.

Continuous administration. Lymph fistula rats allowed to drink only 1 p.c. NaCl solution, with or without food *ad lib.*, drink increasing amounts of the fluid and produce very large volumes of lymph of low protein concentration. With tap water and food *ad lib.*, much smaller volumes of fluid are ingested and the daily production of lymph is much lower (Reinhardt and Bloom, 1949). It seemed of interest, therefore, to compare the rate of thoracic duct lymph production when 0·9 p.c. NaCl and distilled water were administered by gastrostomy at equal rates. The effect on lymph flow of rapid administration of fluid, 4 ml./hr. for 8 hours, is shown in Fig. 1. With saline or water the lymph flow increased by about the same amount in the first hour. Thereafter, the lymph flow increased steadily with saline but diminished during the second half of the infusion period with water, when full diuresis was attained. This difference in pattern was consistent and analysis of the data confirmed its significance statistically. It can also be seen from the 8-hour fluid exchange diagrams in Fig. 1 that 0·9 p.c. NaCl produced a greater total volume of lymph, and a smaller volume of urine was collected than with water. With saline, 21·2±3·5 (S.E.M.) ml. of lymph were collected and 15·8±1·2 ml. with water. The difference was statistically significant ($p < 0·05$). With saline 4·5±1·8 ml. urine was passed compared with a mean volume of 11·4±3·4 ml. during administration of water. The difference was not significant statistically (p approx. 0·1). The after-effects on the lymph flow of rapid saline administration seemed to be greater than those of rapid water infusion. There was not a sufficient number of 16-hour periods of lymph collection for detailed analysis, due to the unsatisfactory nature of the polythene cannulae used in these experiments. However, the results in the experiments which were satisfactory suggest that, of the fluid retained at the end of rapid infusion for 8 hours (see fluid charts, Fig. 1) a greater proportion appeared in the lymph with saline than with water. Thus, in four

16-hour periods after rapid saline infusion, the volume of lymph collected, mean 15·8±1·9 ml., was significantly greater than the volume of saline given, mean 9·3±0·9 ml. (p for difference <0·01). In two 16-hour periods following rapid administration of water, the volume of lymph collected was slightly less than the volume of water given, 13·2 and 8·4 ml. compared with 14·0 and 11·0 ml. respectively. When the volume of urine passed was taken into account, the fluid output in the 24-hour periods was slightly greater than the fluid ingested, whether water or saline.

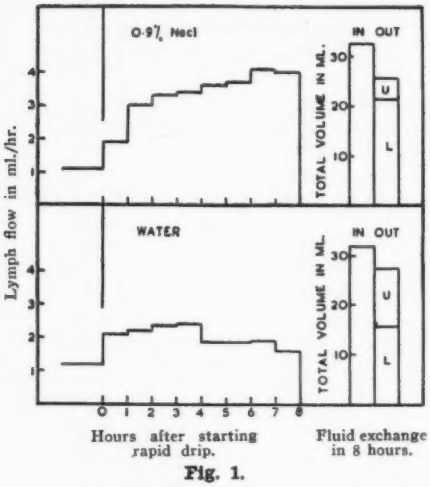

Fig. 1.

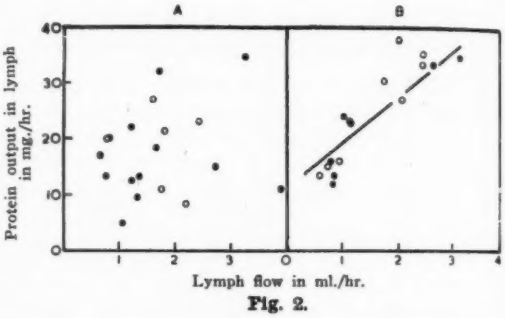

Fig. 2.

Fig. 1. The effect on the hourly production of thoracic duct lymph of administering 0·9 p.c. NaCl solution, upper diagram, and distilled water, lower diagram, at 4 ml./hr. for 8 hours. Each lymph flow diagram is followed by the total fluid exchange in the 8-hour period. U=urine in ml. L=lymph in ml. Mean values for 3 experiments with saline and 4 with water.

Fig. 2. The relationship between protein output in thoracic duct lymph, in mg./hr., and rate of lymph flow, in ml./hr., with high and low salt intake.

(A) Rats on 0·9 p.c. NaCl or distilled water by gastrostomy tube.

(B) Rats with food *ad lib.*, drinking 0·8 p.c. or 0·3 p.c. NaCl solution.

O=low salt intake. ●=high salt intake. Each point represents the value for a single 8 or 16-hour period of lymph collection.

Shrewsbury and Reinhardt (1952) found that rats when they drank large volumes of 0·9 p.c. NaCl solution lost more protein in the thoracic duct lymph, despite the very low concentrations of lymph protein, than rats producing much smaller volumes of lymph of normal protein concentration. The present experiments showed that a "washing out" of protein occurred when the lymph flow was increased either by saline or water administration. In Fig 2 (a), the mean

protein output has been plotted against the mean rate of lymph flow for periods of 8 hours rapid infusion during the day time or 16 hours slow infusion overnight. The protein output increased with lymph flow in much the same way with water as with saline, in individual experiments and in the pooled data from all experiments. Due to the variable fluid balance and food intake in different rats, the overall results did not show a significant linear regression of protein output on lymph flow. In Fig. 2 (b) mean values are similarly plotted for 8 and 16-hour periods in 3 rats without gastrostomies. These rats were allowed food *ad lib.*, 0·8 p.c. NaCl solution to drink for the first 36 hours post operatively and thereafter 0·3 p.c. NaCl solution. Changes in fluid intake were much less sudden and voluntary food intake more consistent than in rats with gastrostomies. The relation between protein output and lymph flow was more clearly similar with high and low salt intakes and the combined data gave a highly significant regression line ($p < 0{\cdot}001$).

Protein output/hr. in mg.=24·2+7·5 (lymph flow/hr. in ml.—1·55). With increased lymph flow, then, the protein concentration was reduced to a similar extent with both water and saline; in neither case was it sufficiently reduced to prevent an increased loss of protein.

Single doses. Continuous gastric infusion was a suitable procedure for investigating the effect of considerable ingested loads of saline or water on lymph flow. However, fluid is normally taken intermittently, and a single period of fluid intake does not usually impose a large fluid or salt load on the body. To approximate more closely to such conditions, 0·9 p.c. NaCl solution or distilled water was administered by gastrostomy tube in single doses of 5 ml. The results in 4 paired experiments in 4 rats are summarized in Fig. 3.

Fig. 3A shows that water produced, almost immediately, a very rapid flow of lymph followed by a rapid return to resting rates of flow. The concentration of protein in the lymph dropped steeply during the period of rapid flow and then was rapidly restored to resting level. With saline, the changes in lymph flow and protein concentration were more gradual and less pronounced. These differences were consistently found. Analysis of variance of the rates of lymph flow during the first, second, third and fourth quarter-hour period after commencing the injection was performed. This showed that the difference in pattern of lymph flow was highly significant (p for interaction, time versus type of fluid, being $<0{\cdot}001$). The total amount of lymph produced in the first hour was not significantly different. If the lymph flow in the hour preceding the injection was used as a baseline, the extra lymph produced in the whole experimental period averaged 2·2 ml. with saline and 1·9 ml. with water. It may be that a larger series of experiments would reveal a slightly greater lymphagogue effect with saline than with water, but the difference could hardly be comparable with that found when large fluid and salt loads were imposed.

Fig. 3B shows that with distilled water the lymph protein was so diluted when the lymph was flowing rapidly that protein output was not significantly increased. With saline, however, increased rates of lymph flow were associated with significantly larger protein outputs. The regression line (p for regression coefficient $<0{\cdot}01$) indicates that with each ml./hr. increase in lymph flow the protein output increased by about 20 p.c. of the resting value. The significance of these findings will be discussed when the mechanism of the increase in lymph flow is considered.

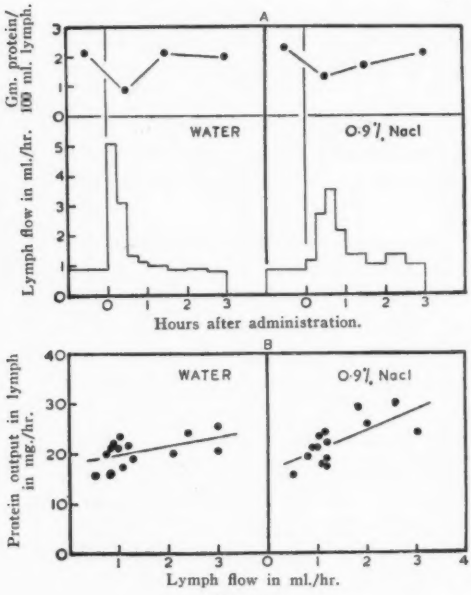

Fig. 3.

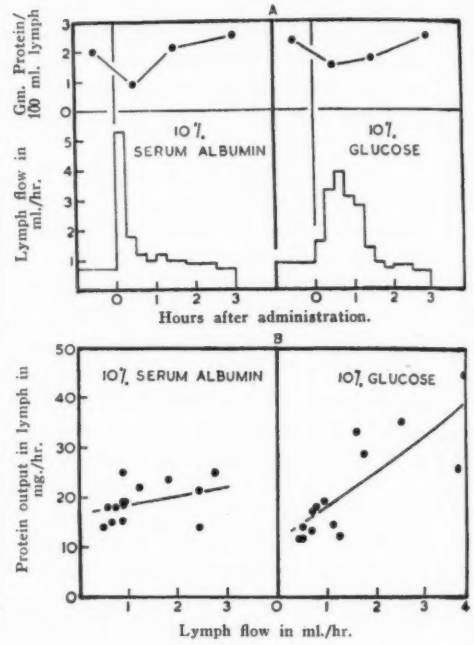

Fig. 4.

Fig. 3. The effect of 5 ml. distilled water and 5 ml. 0·9 p.c. NaCl solution, by gastrostomy, on lymph flow, protein concentration and output of protein in thoracic duct lymph.

(A) Mean rates of flow and protein concentrations in lymph; 4 paired trials in 4 rats.

(B) Relationship between protein output and rate of lymph flow during hourly or 2-hourly periods in the above experiments.

Fig. 4. The effect of 5 ml. 10 p.c. bovine serum albumin solution and 5 ml. 10 p.c. glucose solution in distilled water on lymph flow, protein concentration and output of protein in thoracic duct lymph.

(A) Mean rates of flow and protein concentrations in lymph; 4 paired trials in 4 rats.

(B) Relationship between protein output and rate of lymph flow during hourly or 2-hourly periods in the above experiments.

Effect of foodstuffs in single doses.

Protein and carbohydrate. In 4 paired experiments in 4 rats the effects of 0·5 gm. soluble protein (10 p.c. bovine serum albumin solution) and 0·5 gm. soluble carbohydrate (10 p.c. glucose in water) were compared. The changes in lymph flow and protein concentration in the lymph are summarized in Fig. 4A. With 5 ml. of 10 p.c. albumin solution the results were indistinguishable from those with 5 ml. of distilled water. The extra lymph produced in the whole experimental period (mean 2·0±0·2 ml.) was the same as with water. Similarly, when protein output in the lymph was plotted against rate of lymph flow, Fig. 4B, there was no significant tendency for protein output to increase with lymph flow. Hence there was no evidence that protein, *per se*, affected the rate of flow or protein output in lymph.

With glucose in hypertonic solution, the pattern of lymph flow was consistently very different from that with albumin solution. There was a steady increase in rate for the first half to three-quarter hour and a maintained increase at about 300 p.c. of the resting rate for at least a further half hour. Thereafter the rate decreased, often abruptly, and became steady by the fourth hour. It was by this time significantly lower than the resting value, the mean difference being 0·4±0·1 ml. (p <0·02). This feature did not affect the response to albumin solution, which was the same whether albumin was given before or after glucose. It did, however, make it difficult to estimate the additional lymph produced in the period. The protein output increased significantly with increased rate of lymph flow, Fig. 4B, the regression coefficient in Table 1 indicating that the protein output increased by about 45 p.c. of the fasting value for each ml./hr. increase in rate of flow. Thus, glucose produced changes qualitatively similar to those during absorption of saline. The quantitative differences seemed attributable to the hypertonicity of the glucose solution.

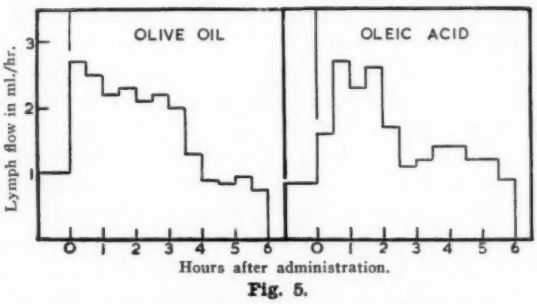

Fig. 5.

Fig. 5. The effect of 0·5 ml. olive oil, left hand diagram, 0·5 ml. oleic acid, right hand diagram, on thoracic duct lymph flow in unanaesthetized rats. The fatty material was washed into the stomach with 4·5 ml. distilled water in each case. Mean values in 3 rats, each of which received both olive oil and oleic acid on different occasions.

Olive oil and oleic acid. A considerable and prolonged increase in lymph flow during absorption of fairly large doses of triglyceride has been reported by

many investigators. Fig. 5 shows that free fatty, oleic acid, produced similar changes to the triglyceride, olive oil. In these experiments 3 rats were used, in each of which, on different days, 0·5 ml. of olive oil and of oleic acid was washed into the stomach with 4·5 ml. of distilled water. The changes in lymph flow were similar in another 6 experiments in 5 rats, in which 0·5 ml. of olive oil or oleic acid was given with varying amounts of water; with the exception that smaller doses of water were followed by smaller increases in lymph flow in the first half hour after injection. Fat appeared in the lymph, in all the rats, from ¾ hour to 1¼ hour after administering the dose. By measurement of lymph flow at quarter-hourly intervals (see Fig. 6) it was usually possible to demonstrate that a secondary increase of lymph flow commenced at about this time. Fig. 5 suggests that the triglyceride produced a different pattern of lymph flow from the free fatty acid. With olive oil the rate of lymph flow returned to resting values within about 4 hours. With oleic acid a slightly increased rate of flow persisted until the end of the 5th to 7th hour after injection. Further work will be necessary to determine the significance of these differences, which also appeared in experiments with smaller doses of water but the same dose of fat. Experiments in progress suggest that they are related to a difference in the pattern of fat output in the lymph after olive oil and oleic acid. It should be mentioned that the extra amount of lymph produced in the whole experimental period after olive oil and oleic acid was the same. The mean values were 4·6 and 4·7 ml. respectively, much greater than with similar doses of fluid and other foodstuffs.

Absorption of olive oil or oleic acid seemed to produce a more marked increase in protein output in the lymph than any other substances tested. For

TABLE 1.

The linear regression of protein output on lymph flow after single doses of fluid (5 ml.) with or without foodstuffs (0·5 gm.).

Material given	No. of observations	Linear regression formula[1]	"p" for significance of regression coefficient from 0
Water	14	$Y=20·5+ 1·7\ (X—1·4)$	Not significant
0·9 p.c. NaCl solution	14	$Y=22·1+ 4·1\ (X—1·4)$	$<0·01$
10 p.c. glucose in water	14	$Y=21·5+ 7·4\ (X—1·5)$	$<0·001$
10 p.c. serum albumin in water	15	$Y=18·8+ 1·9\ (X—1·3)$	Not significant
Olive oil and oleic acid[2]	14	$Y=25·9+ 9·6\ (X—1·8)$	$<0·001$
Olive oil and oleic acid[2]	11[3]	$Y=25·7+10·2\ (X—1·7)$	$<0·001$
Olive oil and oleic acid	14	$Y=21·2+ 6·4\ (X—1·4)$	$<0·1$ not significant
Olive oil and oleic acid	11[3]	$Y=21·5+15·0\ (X—1·2)$	$<0·001$
Skim milk	34	$Y=18·3+10·5\ (X—1·2)$	$<0·001$
Full cream milk	16	$Y=17·2+ 7·5\ (X—1·3)$	$<0·001$

[1]Where Y=protein output in lymph, in mg./hr.
X=rate of lymph flow, in ml./hr.

[2]With small amounts of water.

[3]After excluding observations in first hour after administration.

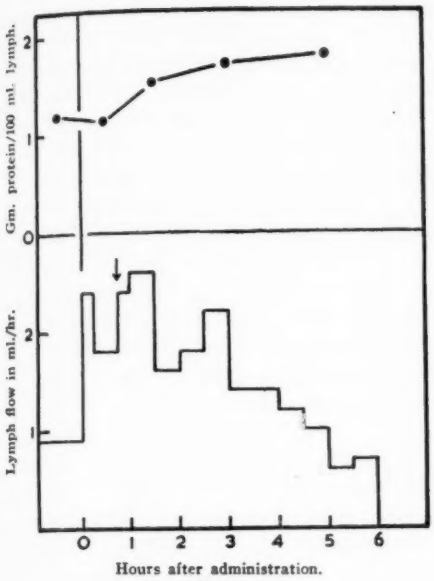

Fig. 6.

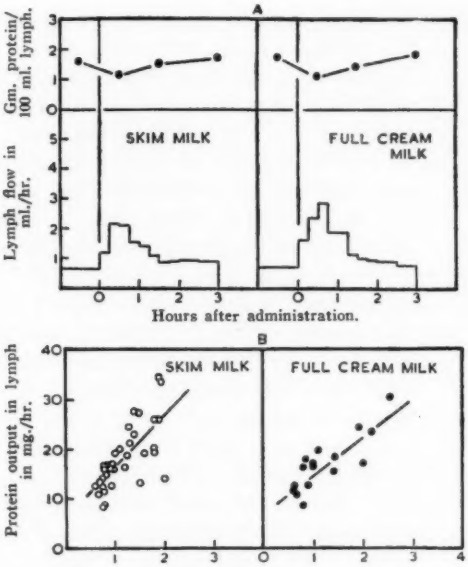

Fig. 7.

Fig. 6. A typical experiment, Rat 32, in which rapid flow of thoracic duct lymph during fat absorption was not accompanied by lowered lymph protein concentrations. At 0 hours, 0·5 ml. olive oil was washed into the stomach with 2 ml. water. The rise and decline of flow in the first ¾-hour were associated with absorption of the water. At the time indicated by an arrow the lymph became milky and the flow increased again.

Fig. 7. The effect of 5 ml. 10 p.c. (W/V) skim milk powder and full cream milk powder, both in 0·5 p.c. sodium citrate solution, on lymph flow, protein concentration and output of protein in thoracic duct lymph.

(A) Mean rates of lymph flow and protein concentrations in lymph; 4 paired trials in 4 rats.

(B) Relationship between protein output in lymph and rate of flow in 9 trials in 6 rats with skim milk (34 observations) and 4 trials in 4 rats (16 observations) with full cream milk.

example, when the early dilution of lymph was minimized by administering only a small volume of water the protein concentration remained the same, or even increased, despite a considerable increase in lymph flow. A typical experiment of this sort is depicted in Fig. 6. When protein output was plotted against rate of lymph flow, with small doses of water it was found (Table 1) that the linear regression had a highly significant slope ($p < 0{\cdot}001$). The regression coefficient indicated that each increment in lymph flow of 1 ml./hr. was accompanied by an increase in protein output of about 60 p.c. of the resting value. With larger volumes of fluid, 5 ml., the added water depressed the lymph protein concentration unduly in the period immediately after administration. This was indicated

by finding that the slope of the regression line was not significant when the values for the first hour after injection were included, but highly significant ($p < 0{\cdot}001$) if these were excluded. The regression coefficient then indicated that the protein output increased by over 80 p.c. of the resting value when the lymph flow increased by 1 ml./hr. This estimate was not very reliable (the standard deviation of coefficient being 20 p.c. of its mean value). With small volumes of fluid, exclusion of the values for the first hour did not alter the slope of the regression line appreciably. The changes in protein concentration in the lymph with fat absorption do not suggest that a gross increase in capillary permeability occurred.

Skim milk and full cream milk. The dose of fat or fatty acid given in the experiments described above was a fairly large one, 2 to 2½ gm./kg. body weight, which is about equal to the daily intake of fat on a diet of rat cubes. To see if a small dose of fat would influence the lymph flow after a mixed meal, the effects of 0·5 gm. full cream milk powder, containing about one-quarter the amount of fat previously given, and of 0·5 gm. skim milk powder were compared. Both substances were given in 5 ml. distilled water. Fig. 7A summarizes the changes in lymph flow and protein concentration in 4 paired experiments in 4 rats. All these experiments were performed on the day following operation. Full cream milk produced a somewhat greater lymph flow than skim milk in the first 2 hours after administration. Analysis of variance of the data confirmed the significance of this finding statistically ($p < 0{\cdot}001$). The difference was not large. With both full cream and skim milk, the lymph flow sometimes remained a little above the resting value even in the third and fourth hours. The mean total extra volume of lymph produced in the whole period was 2·2 ml. with full cream milk and 1·85 ml. with skim milk. The pattern of lymph flow after full cream milk was not significantly different from that after skim milk. Thus, the absorption of a single small dose of milk fat, as full cream milk, had a very small effect on lymph flow.

Table 1 and Fig. 7B show that a similar increase in protein output with increase in lymph flow occurred after full cream and skim milk. The regression coefficients indicated an increase in protein output by about 55 and 75 p.c., respectively, of the resting value when lymph flow increased by 1 ml./hr. The results with skim milk were the more variable, and an extra 5 experiments were performed in 2 other rats to obtain a highly significant regression line. The changes in lymph flow in these experiments were similar to those in Fig. 7A.

Suspended solid food. In 3 experiments in 2 rats, 0·5 gm. of suspended rat cubes in 5 ml. distilled water was given through a wide gastrostomy tube. The changes in lymph flow and protein concentration in the lymph were similar to those after fluid alone. There was no evidence of an increased lymph flow in the second or third hour associated with absorption of the products of digestion. It did not appear, therefore, that a larger series of experiments of this type would be profitable.

DISCUSSION.

The effect of fluid and electrolytes. There is general agreement that giving fluid by the intestinal route increases the flow of thoracic duct lymph, but the relative efficiency of water and saline as lymphagogues appears to be determined by the manner of administration. Watkins and Fulton (1938) gave about 30 ml./kg. body weight by stomach tube to anaesthetized dogs. They found that the average lymph flow in the first 40 to 65 minutes after administration was greater with water than with 0·9 p.c. NaCl solution. Glenn, Cresson, Bauer, Goldstein, Hoffman and Healey (1949) measured the thoracic duct lymph flow in unanaesthetized dogs which received in 9 to 15 hours a total of 100 to 150 ml. fluid/kg. body weight. They found that 5 p.c. glucose in water produced a moderate increase in lymph flow, initially, and a relative diuresis, while 5 p.c. glucose in 0·85 p.c. NaCl solution produced a sustained and continued increase in lymph flow with a low urinary output. With smaller doses there was no appreciable difference between glucose-water and glucose-saline.

Experiments with unanaesthetized rats have confirmed the part played by the degree as well as the type of fluid loading. Thus, there was no detectable difference in the total amount of extra lymph produced when distilled water and 0·9 p.c. NaCl solution were given in a single dose of 20 to 25 ml./kg. body weight. On the other hand, 0·9 p.c. NaCl solution produced a greater flow of lymph than water when both were given in doses of about 150 ml./kg. body weight, by continuous drip at about 20 ml./kg./hr. The differences in pattern of lymph flow after single doses or during continuous administration, and the changes in protein output in the lymph with increased flow, suggested the mechanisms which might be involved.

The intestinal epithelium is virtually impermeable to protein and there seems little doubt that the ultimate source of the protein in intestinal lymph is the capillary filtrate. An increase in lymph flow associated with rapid expansion and dilution of the interstitial fluid of the villi by absorbed solutions should be accompanied, therefore, by a low concentration of protein and little or no change in protein output in the lymph. Slow expansion by slowly absorbed solutions should give time for passage of some protein through the capillary walls, and output of protein in the lymph should increase, but to a lesser extent than the flow. This should also apply if the tissue spaces are expanded by capillary filtrate, when the output of protein might be somewhat greater. Finally, with an increased lymph flow due to increased propulsion of lymph an unchanged protein concentration, and increase in protein output to the same extent as lymph flow, would be expected.

On the above basis, obviously over-simplified, the following explanation of the changes after a single dose of fluid (Figs. 3 and 4) may be put forward. Water was rapidly absorbed. It therefore caused considerable dilution of intes-

tinal extracellular fluid and an initially rapid flow of lymph of low protein concentration, with rapid return to resting values. Saline was more slowly absorbed. The changes in lymph flow were therefore more gradual, the dilution of interstitial fluid and lymph was less and the output of protein greater than with water, although the total extra volume of lymph produced was about the same. Thus, with single doses of water and saline, and probably in the case of hypertonic glucose solution as well, the fluid modified the flow and protein concentration of the lymph in the course of absorption. When saline and water were administered continuously (Fig. 1) the degree of expansion of the interstitial fluids in the intestinal mucosa was also influenced by progressive changes in the load of fluid retained by the body as a whole. That is, the capillary filtrate played a more important part in augmenting the lymph flow. This was indicated by the continued increase in lymph flow during saline administration, while with water the flow increased only during the early stages and decreased after diuresis was fully established. A decreased flow of thoracic duct lymph during a diminishing body load of water, as a result of diuresis, was demonstrated by Watkins and Fulton (1938). Dependence of lymph flow on capillary filtration when large doses of water and saline were given over a fairly long period was also indicated by the similarity in protein output with the two fluids (Fig. 2). If saline had produced a greater total volume of lymph because more of it passed from interstitial fluid into lymphatics during absorption, a more dilute lymph and a lower protein output than with water would have been expected. The protein concentration in the lymph was low in nearly all experiments with fluid, electrolytes and foodstuffs—usually about 2 gm. protein/100 ml. This was almost certainly due to washing out of protein in the extracellular fluid by the large volumes of fluid given to maintain hydration and lymph flow. Plasma protein concentrations were estimated in some rats killed after infusion of fluid and collection of lymph for various periods. The plasma protein concentration was usually low, due to protein loss from the lymph fistula and low food intake, but the hypoproteinaemia did not appear to be mainly responsible for the low lymph protein concentrations. The latter appeared to depend more on the rate of fluid administration in the periods before the lymph sample was collected. It would not be advisable, therefore, to compare too closely protein turnovers in the lymph on different experimental days and in different animals. However, the fact that there was no difference attributable to continuous infusion of water compared with saline is significant, since conditions were purely comparable in these experiments. It also seems legitimate to compare changes when substances were given in successive periods on the same experimental day, the order of administration being reversed each time the experiment was repeated.

Foodstuffs. The effect of digestive processes on lymph flow cannot be assessed with certainty, due to the fluid given with all meals. Protein in solution

or a suspended mixed food, rat cubes, had an effect similar to water alone. This may indicate that digestion and trituration of the food had little effect.

During absorption of the three main dietary constituents there was clear evidence for an increase in lymph flow only in the case of fat. With glucose, the hypertonicity of the solution given seemed to account for the changes, but the effects of fluid absorption may have masked those of glucose. The absence of an increased flow in the second and subsequent hours after feeding suspended rat nuts, when digestion products would have been available more slowly for absorption, suggested that neither carbohydrate nor protein has a pronounced effect. This was supported in the case of protein by the results after feeding bovine albumin solution. The fact that fat absorption increases lymph flow in the unanaesthetized animal is well known and will not be discussed until substantial evidence concerning the mechanism can be brought forward. It was, however, interesting to observe that the lymph became very milky after oleic acid as well as after olive oil, and that the increase in lymph production was of the same order. This was not in accord with Frazer's observation, in 1943, that the contents of lymphatics at the root of the mesentery did not appear milky at autopsy in rats fed with oleic acid. Frazer (1943) also found that the fat soluble dye, Sudan IV, appeared in the liver in rats fed with dyed oleic acid and in the fat depots after dyed olive oil. He inferred that oleic acid, carrying dye with it, was absorbed into the portal blood stream and not into the lymphatics. Experiments in which the amount of dye recovered in the lymph has been measured will be reported at a later date. They have not, up to the present, revealed a significant difference between dyed olive oil and dyed oleic acid.

In discussing changes in flow, it has been implied that changes in the rate of formation of lymph are mainly responsible. The large volume of lymph collected during fat absorption and the changes in protein concentration after fluid administration are not consistent with increased propulsion of preformed lymph, such as occurs with abdominal massage. The assumption that the extra lymph came mainly from the intestinal lymphatics requires some comment. It is very unlikely that lymph from the hind limbs contributed significantly to the flow in fasting or fed animals, which remained quite still for long periods. The liver contributes a much larger quota to the thoracic duct lymph flow than the skeletal structures, but it is unlikely that it played a large part in the observed increases in flow. In rats with food and 0·2 p.c. NaCl solution *ad lib.*, Mann and Higgins (1950) found that the liver and intestinal lymph flow were 2·0 and 25·1 ml. per day respectively. However, in dogs quarter to half of the thoracic duct lymph is said to come from the liver (Cain, Grindlay, Bollman, Flock and Mann, 1947).

It is difficult to estimate the volume of thoracic duct lymph produced per day as a result of the voluntary ingestion of food, since lymph fistula animals are not in metabolic balance. Results, such as those of Reinhardt and Bloom

(1949) and Shrewsbury and Reinhardt (1952), with rats on different regimes suggest that the daily output of lymph may be 50 to 100 p.c. greater in fed than in fasting animals. With regard to the effect of a single meal, Crandall, Barker and Graham (1943) found that, in man, the lymph flow increased to about 3 times its resting value immediately after a heavy meal, containing fat, and returned to normal within 85 minutes. A light meal without fat had practically no effect. Fat alone, olive oil in a dose of 1·25 ml./kg., had a considerable and prolonged effect, concurrent with its absorption into the lymph. These experiments were performed with a patient who had a thoracic duct fistula in the neck. Cain *et al.* (1947) observed, in unanaesthetized dogs, an increased flow of thoracic duct lymph lasting not less than 5 hours. The type of meal did not appear to influence the results. The liver lymph flow increased in about the same proportion as the thoracic duct lymph flow, approximately by 100 p.c. of the resting value. The proportion of the increased lymph flow due to fluid and electrolytes taken with the food, and in it, may be less than the present experiments with rats might suggest. Rapid absorption of fluid taken in the fasting state might cause a greater disturbance in lymph flow than the fluid in a meal. The dose of fluid per kilogram body weight given to rats was also larger than that usually taken by man at meal times. However, apart from fluid, fat was the only constituent of a meal which, when taken alone, appeared to increase the flow considerably in rats. In view of the small effect of fat in a mixed food such as full cream milk, and of Cain *et al.* results in dogs, it will be necessary to give food with minimal amounts of fluid before one can be confident that digestion and absorption of constituents of a meal other than fat have little effect on thoracic duct lymph flow.

SUMMARY.

The effect on lymph flow and protein concentration of fluid, electrolyte and food, given by gastrostomy tube, was investigated in unanaesthetized rats with an indwelling plastic cannula in the cisterna chyli.

When fluid was given rapidly and continuously in large loses, about 20 ml./kg./hr. for 8 hours, more lymph was produced and less urine passed during adminstration of 0·9 p.c. NaCl solution than when distilled water was given. With saline the lymph flow increased progressively during the 8-hour period, with water the rate of flow diminished once full diuresis was established. Protein output in the lymph increased as the lymph flow accelerated, in much the same fashion with saline as with water. It is suggested that the more rapid lymph flow with saline was due to a greater load of retained fluid increasing capillary filtration of both fluid and protein.

When fluid was given in single doses of 16 to 20 ml./kg., the same extra volume of lymph was produced with water as with saline. The pattern of increased lymph flow was different, maximal rates of flow being attained in the

first quarter hour after water, with a rapid decline, while the increase and decrease were more gradual after saline. The lymph protein was diluted more after water. It is suggested that, after a single, fairly large dose of fluid, the dilution and expansion of the mucosal extracellular fluid during absorption is mainly responsible for the increased lymph flow.

Regarding the effect of foodstuffs, when 0·5 gm. protein, serum albumin, or carbohydrate, glucose, was dissolved in 16 to 20 ml. fluid/kg. body weight the effect on lymph flow seemed attributable to absorption of the fluid. With the hypotonic albumin solution the changes resembled those after water, while the hypertonic glucose solution had an effect similar to saline. Triglyceride, olive oil, or fatty acid, oleic acid, produced a prolonged increase in lymph flow, concurrent with fat absorption, when given in large doses, 2 to 2½ ml./kg. body weight, in small or large volumes of water. Smaller doses of fat, about 0·5 ml./kg. body weight, given as full cream milk did not have an effect very different from skim milk.

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AN ANALYSIS OF FACTORS AFFECTING LYMPH FLOW AND PROTEIN COMPOSITION DURING GASTRIC ABSORPTION OF FOOD AND FLUIDS, AND DURING INTRAVENOUS INFUSION[1]

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In investigations of the mechanisms concerned in filtration through the capillary membrane, important information concerning the amount and protein composition of the capillary filtrate cannot be obtained directly because this fluid cannot normally be collected. The only part of the extravascular fluid that can be collected under normal circumstances is lymph. When filtration through the capillaries exceeds reabsorption in any area, the rise in tissue fluid will increase the tissue pressure which, in turn, will give rise to an increase in lymph flow. An equilibrium will be reached when all the excess fluid filtered will enter the lymphatics and be returned to the blood stream as lymph. Thus, although there may be lags in the changes in lymph flow because of variations in tissue fluid volume, the lymph over a fairly long period represents the excess of filtration over reabsorption through the capillary bed.

In animals where lymph is drained off for considerable periods of time the total protein output in the lymph will not *per se* be an index of the protein leakage from the capillaries. Lymph protein comprises in part protein from the extravascular protein pool and in part capillary filtrate, the concentration of the latter being considerably below the former. When there is an excess of fluid filtration over reabsorption the protein output will for some time consist of both filtrate protein and extravascular protein, and the latter will tend to become reduced in amount. If, for example, conditions can be attained where reabsorption is negligible, then the concentration of protein in the lymph should ultimately approximate that in the capillary filtrate.

The several unknown factors in these fluid exchanges, viz., the volume and protein composition of the capillary filtrate, the volume of reabsorbed fluid and the size of the extravascular protein pool, make it difficult to assess the significance of the proteins and of the permeability of the capillary membrane

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[2]George Aitken Pastoral Research Trust Scholar.

to proteins in the fluid balance of the body. In the alimentary tract, where there is a very large turnover of plasma protein, another unknown factor, direct absorption of water and of other substances into the lymphatics from the lumen of the gut, further complicates the picture (Simmonds, 1954). The present study is an attempt to evaluate the changes that occur in the thoracic duct lymph flow and protein composition during absorption of food and fluids from the gut and during large intravenous infusions of Ringer-Locke solution. In the latter circumstances reabsorption of fluid into the blood capillaries is considerably reduced and filtration increased.

METHODS.

Animals. The lymphatic experiments were carried out on 58 cats whose weights varied from about 2 to 4 kg. Anaesthesia was effected with Nembutal (Abbott) given intravenously. Some additional experiments on the effect of intravenous infusions of Ringer-Locke solution were carried out on a group of 20 intact, unanaesthetized rabbits varying in weight from 1 to 2·5 kg.

Lymphatic cannulation. Thoracic duct cannulation was carried out at the jugular venous confluence, using a glass cannula in most instances and a polyethylene cannula in a few experiments. Lymph was collected with a Pasteur pipette into a heparinized vessel, or when a polyethylene cannula was used, the lymph flowed directly into the vessel. All the lymph flow rates reported represent spontaneous flow.

Blood samples. Polyethylene catheters were inserted into the femoral artery, and via the saphenous vein to the lower end of the inferior vena cava. Substances to be injected or infused were administered through the venous catheter, and samples for analysis were obtained from the arterial catheter, or from an arterial cannula inserted in the femoral artery.

Infusion of fluids. The electrolyte solution used in these experiments consisted of sterile Ringer-Locke solution. This was administered, in the case of the cats, through the venous catheter at a constant flow rate by means of a standard Murphy's drip apparatus. The time of administration was either 3 or 5 hours in different experiments. In the experiments on rabbits, the drip was connected to a needle in the lateral ear vein. The enteral administration of fluids was effected through a rubber stomach tube and this was connected to a drip bottle and fluid administered at a standard rate of 40 ml./kg./hour. In all experiments the amount of urine produced following the various infusions was carefully noted.

Protein determinations. Total protein and N.P.N. estimations were carried out on plasma and lymph by micro-kjeldahl digestion, nesslerization and estimation with a Hilger "Spekker" absorptiometer. Albumin was determined by the method of Campbell and Hanna (1937) using 21 p.c. sodium sulphite as a precipitant. Globulin was estimated by subtracting the albumin figure from the total protein.

Electrophoresis. Electrophoretic patterns were done on both lymph and plasma samples. The method used was that described by Flynn and de Mayo (1951) using a broad strip micro-electrophoresis technique on filter paper. The samples were run for 17½ hours at a potential of 125 V in a barbiturate buffer pH 8·6. Protein patterns were stained with bromophenol blue mercuric iodine stain and final colour development achieved with ammonia. The filter paper strips were then cleared in liquid paraffin and air bubbles removed by exhausting in a vacuum oven for 15 minutes. The differences in optical density along the pattern were then measured by means of a photoelectric scanner.

Blood volume determinations. This was done using the Evans blue (T1824) dye-haema-

tocrit method. Samples were obtained at 15 and 30 minutes following injection of an aqueous solution of dye, 600 mg./litre. The concentration of the dye was measured by extracting the dye from the plasma, using a modification of the method of Allen (1951). The concentration at zero time was obtained by linear extrapolation of the determined points. All haematocrits were done in duplicate and spun for 55 minutes at 3,000 revs./minute. In some cats blood and plasma volumes were measured after an initial period of 1½ hours lymph collection, whilst in others they were measured before cannulating the thoracic duct. There was no significant difference in the two groups. The average results from 37 cats were 58 ml./kg. for blood volume, and 35 ml./kg. for plasma volume.

Extraction of T1824 from lipaemic lymph. The original method of Allen (1951) was found to be not entirely satisfactory in extracting the dye T1824 from very fatty lymphs. The following modification of the original method has been found to give excellent results. To 1 ml. of lymph is added 0·5 ml. of sodium laurol sulphate, and the mixture shaken and allowed to stand for 5 minutes. It is then shaken and poured on to a 1 cm. high cellulose column; the dye is adsorbed on this by filtering through with gentle suction in about 10 minutes. The column is then washed with 6 ml. of 0·9 p.c. NaCl, the latter being poured on top of it and sucked through. Following this, 3 washes of 3 ml. each of ether are then sucked through; the ether does not elute any of the T1824 from the column. Two further washes with 3 ml. of 0·9 p.c. NaCl are then carried out to remove any of the remaining ether. The dye is then eluted with a 1:1 acetone:distilled water mixture into a 10 ml. volumetric flask, the latter process lasting about 45 to 60 minutes. In this way completely clear samples of T1824 in acetone water can be obtained. The percentage extraction is the same as that of the original Allen method, 96 to 98 p.c. of the dye present on the adsorption column being recovered. The concentration of the dye was determined at a wavelength of 620 mμ with a Beckman spectrophotometer.

Arterial pressures were measured through the femoral arterial catheter on a mercury manometer.

Venous pressure. In many experiments the pressure changes in the lower inferior vena cava resulting from transfusion were measured through the indwelling polyethylene catheter, using the method of Burch and Winsor (1943). In a few experiments these pressures were compared to pressure changes in the portal system during transfusion and the following operative procedure was used. A small transverse left upper abdominal incision was made and the spleen immobilized; a polyethylene catheter was inserted into the main splenic vein and pushed along the vein for a distance of about 3 cm. The splenic vessels were then tied and splenectomy performed and the abdominal wound closed. The pressure changes measured through the catheter thus represent changes in the pressure in the portal system excluding the spleen. In the rabbit experiments the venous pressure was recorded in the ear vein; this has been shown during intravenous infusion to parallel closely changes in the jugular venous pressure, though the ear vein figures give about 6 to 7 cm. systematically higher readings than the jugular figures (Korner, unpublished data).

Statistical analysis. The statistical procedures used are fully described by Fisher (1952) and Mather (1951).

RESULTS.

Effect of intravenous transfusion of Ringer-Locke solution on lymph flow and plasma-lymph protein dynamics.

Effect of transfusion volume, respiratory factors and changes in filtration pressure on lymph flow. Fig. 1 shows the result of a typical experiment in which the animal was transfused with Ringer-Locke solution for 5 hours. The

lymph flow rose continuously for the first three hours of the experiment and then reached a fairly steady plateau level. This rise in lymph flow was accompanied by a rise in lower inferior vena caval pressure, which, whilst small, closely parallelled the changes in lymph flow. There was no significant change in the mean femoral arterial pressure. After the transfusion ceased the venous pressure returned rapidly to the resting value and the lymph flow returned to the pre-infusion level after 1 to 2 hours.

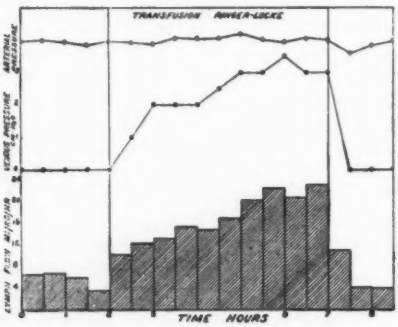

Fig. 1.

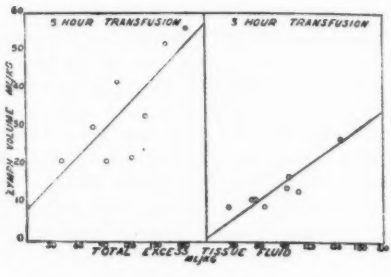

Fig. 2.

Fig. 1. Effect of intravenous infusion of Ringer-Locke (120 ml./kg./5 hr.) on rate of lymph flow (ml./kg./hr.), venous pressure (cm. H_2O) and arterial pressure (mm. Hg.).

Fig. 2. Relation between total excess tissue fluid (ml./kg.) and volume of lymph produced (ml./kg.) during intravenous administration of Ringer-Locke for 5 and 3 hours.

Fig. 2 shows the relation between the total excess tissue fluid (i.e., volume of Ringer-Locke transfused minus urine produced minus excess fluid in plasma) retained by the animal, and the volume of thoracic duct lymph collected in 9 five-hour experiments and 8 three-hour experiments. In the five-hour experiments, the excess amount of lymph produced by the transfusion amounted to approximately 24 p.c. of the excess tissue fluid retained, while in the three-hour experiments it amounted to only about 13 p.c. The reason for this difference is evident from Fig. 1, which shows that as the tissue fluid volume expands during transfusion, lymph flow increases until at 3 hours an equilibrium is reached. When the lymph flow reaches a plateau value in this type of experiment, the volume of tissue fluid will have reached a steady state and the excess lymph production per unit excess of tissue fluid will be a measure of the net filtration into the thoracic duct bed. The three-hour transfusion figures give a greater underestimate of the size of the net capillary filtration volume during transfusion than do the five-hour figures. The capillary filtration volume is, under transfusion conditions, probably somewhat greater than the estimate obtained from the five-

hour figures. Under these circumstances about 30 p.c. of the total net capillary filtration occurs in the region drained by the thoracic duct.

It is well known that, in certain conditions, increased activity of the respiratory muscles can produce a rise in lymph flow. In the present experiments there was no systematic change in respiratory minute volume and rate during the intravenous infusions. It follows, therefore, that respiratory factors do not account for the characteristic flow patterns and increased lymph volume during transfusion.

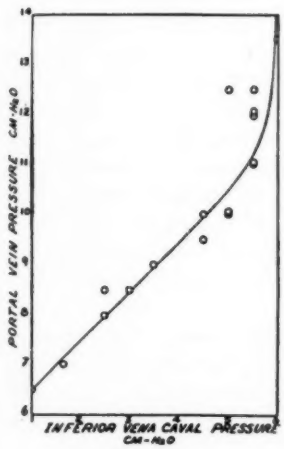

Fig. 3.

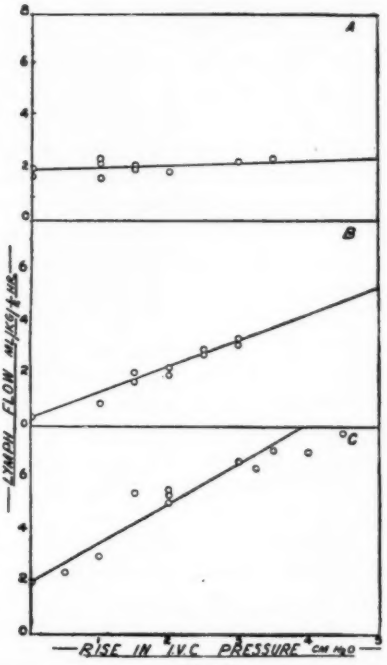

Fig. 4.

Fig. 3. Simultaneous measurement of changes in portal vein and lower inferior vena caval pressure during intravenous administration of Ringer-Locke. The reference level was 4 cm. above the point of the vena caval polythene cannula.

Fig. 4. Relation between rise in I.V.C. pressure (cm. H_2O) and lymph flow (ml./kg./½ hr.) in 3 individual experiments with different net infusions.

A=net infusion of 40 ml./kg./5 hr.
B=net infusion of 120 ml./kg./5 hr.
C=net infusion of 180 ml./kg./5 hr.

Intravenous administration of Ringer-Locke solution produced a significant rise in venous pressure in the inferior vena cava. Fig. 3 shows in a typical experiment that changes in pressure in the lower inferior vena cava and portal

vein ran parallel, though the portal pressure was at a higher absolute level. Thus changes in the lower inferior vena caval pressure afford a guide to changes in portal venous pressure during transfusion. In all the experiments there was a fall in plasma protein concentration, which was not very different with moderate and large transfusions (range of total excess tissue fluid retained from 70 to 180 ml./kg./5 hr.). The greatest drop in plasma protein concentration occurred during the first 2 to 3 hours of transfusion coinciding with the maximum rise of venous pressure. Although on an average the plasma protein concentration fell from 6·7 to 4·9 gm. p.c., the lymph proteins fell from 4·2 to 2·2 gm. p.c. If the fall in the level of lymph proteins is considered to reflect the fall in average tissue fluid protein concentration, the effective osmotic pressure of the proteins will not be decreased. The change in venous pressure has therefore been used as an index of change in the filtration pressure. The average absolute values for lower vena caval pressures at rest varied between 2 to 5 cm. of H_2O, this corresponding to portal pressures of about 8 to 12 cm. H_2O. The rise of venous pressure during the transfusion experiments was about 3 to 6 cm. of H_2O, corresponding roughly to portal pressures of 11 to 18 cm. H_2O (Fig. 3). In Fig. 4 the lymph flow (ml./kg./$\frac{1}{2}$ hr.) has been plotted against the rise in lower inferior vena caval pressure. It may be seen that there is a relationship between lymph flow and rise in venous pressure in all the three experiments shown. The slope expressing this relationship varies, however, with the degree of total excess tissue fluid retained by the animal. Unit increment of venous pressure leads to a smaller rise in lymph flow in experiment 4A, where the fluid retained was less than in experiments 4B and 4C. This graded effect was noted in all eight experiments where this point was investigated, suggesting that the larger the volume of excess tissue fluid the more does the rise of venous pressure affect the flow of lymph.

Lymph protein composition during intravenous transfusion. The influence of changes in plasma protein concentration and rate of lymph flow on the thoracic duct protein and albumin concentration was studied during the intravenous transfusion experiments. Lymph and plasma samples used in this analysis were taken only during steady state conditions, i.e., after 2 to 3 hours transfusion, when the lymph flow had become fairly constant. The results have been summarized in Table 1. These show that the thoracic duct protein concentration during Ringer-Locke transfusion was related to the plasma protein concentration at that time and was independent of the rate of lymph flow. The same results were obtained from the analysis of factors influencing the thoracic duct albumin concentration.

Wasserman and Mayerson (1952) have shown that albumin leaves the capillaries about 1·6 times faster than globulin. Our results substantially agree with these findings. In 10 experiments on resting cats the average plasma protein

TABLE 1.

Regression of plasma protein and albumin concentration and lymph flow on thoracic duct protein and albumin concentration during intravenous Ringer-Locke infusion.

Experiment	No. of results	Multiple regression equation	First regression coefficient	Signif. of first regression coefficient	Second regression coefficient	Signif. of second regression coefficient
I.V. Ringer-Locke	32	T.D.P.C.=2·22+0·557 (P.P.C.—4·9)—0·031 (L.F.—8·7)	0·557± 0·043	Signif. $p<0·001$	0·031± 0·022	Not Signif.
I.V. Ringer-Locke	12	T.D.A.C.=1·65+0·849 (P.A.C.—3·0)—0·022 (L.F.—7·8)	0·849± 0·303	Signif. $p=0·02$	0·022± 0·027	Not Signif.

T.D.P.C. is thoracic duct protein concentration gm. p.c.
T.D.A.C. is thoracic duct albumin concentration gm. p.c.
P.P.C. is plasma protein concentration gm. p.c.
P.A.C. is plasma albumin concentration gm. p.c.
L.F. is lymph flow ml./kg./hr.

concentration was 7·3 gm. p.c., and the plasma albumin concentration was 3·8 gm. p.c. The A/G ratio in plasma was thus 1·09. In corresponding lymph samples the average total protein concentration was 5·1 gm. p.c. with an albumin concentration of 3 gm. p.c. The A/G ratio in resting lymph was thus 1·43, the difference being statistically significant. During Ringer-Locke transfusion the A/G ratio in the lymph was rather more variable than in resting cats, but the mean of 10 experiments was still about the same (1·5). Whilst transfusion increased the rate of protein filtration, the proportional rate of movement of albumin to globulin remained at about the resting value.

Plasma-lymph protein exchange during transfusion. Fig. 5 shows the results of four typical experiments where the specific activities (dye concentration/protein concentration) have been plotted during transfusion. Fig. 5A and 5B show that equilibrium between plasma and lymph was not attained during five hours when only small transfusions were given. With larger net infusions (Fig. 5C and 5D) the specific activities of plasma and lymph became equal after 1½ to 3 hours transfusion. Large intravenous infusions of Ringer-Locke solution thus greatly increase the rate of protein exchange between plasma and lymph. The specific activities of lymph and plasma were also shown to be similar for albumin and total protein, and both estimates give similar equilibrium times.

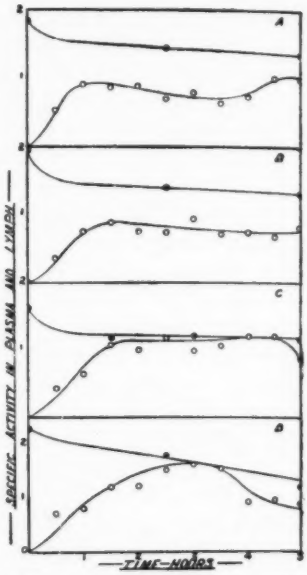

Fig. 5. Specific activity (T1824 concentration/plasma protein concentration) in plasma and lymph with different amounts of I.V. Ringer-Locke.
A=no transfusion.
B=net infusion of 40 ml./kg./5 hr.
C=net infusion of 120 ml./kg./5 hr.
D=net infusion of 180 ml./kg./5 hr.

The average loss of total plasma protein in the course of 8 five-hour transfusion experiments was 25±3·6 p.c. of the total circulating amount at the beginning of the experiment. The loss of T1824 was 50±3·4 p.c. of the initial circulating amount. There is good evidence that T1824 is mainly albumin bound (Rawson, 1943; Allen and Orahovats, 1951), and that the dye-protein complex is very rapidly formed and is quite stable for at least one week (Freinkel, Schreiner and Athens, 1953). In the present experiments the discrepancy between the loss of total plasma protein and the loss of T1824 labelled protein may best be explained by postulating the mobilization of new unlabelled protein into the circulation. The mean total circulating plasma protein and albumin were 7·0 and 3·5 gm. respectively. Since the dye is mainly attached to albumin

which passes through the capillaries 1·6 times more rapidly than globulin, the loss of all the circulating dye would be accompanied by a plasma protein loss of about 5·7 gm. In the present experiments an average of 50 p.c. of the amount of dye present at the start was lost during the five-hour experiments. This would correspond to a total protein loss of 2·8 gm. The actual average loss was, however, only 1·4 gm. Thus, about 1·4 gm. of new protein entered the circulation during the five hours of transfusion. The detailed balance charts of two additional three-hour transfusion experiments are shown in Table 2 to illustrate the above point.

TABLE 2.

Protein and 1824 balance chart in plasma during intravenous infusion of Ringer-Locke solution in two experiments.

	Experiment (1)		Experiment (2)	
	Start	End	Start	End
Total circulating protein	5·80 gm.	4·88 gm.	7·20 gm.	4·07 gm.
Total circulating albumin	3·80 gm.	3·04 gm.	3·70 gm.	2·76 gm.
Total circulating T1824	1·463 mg.	0·903 mg.	2·707 mg.	1·456 mg.
T1824 loss during transfusion		0·560 mg.		1·251 mg.
Expected albumin loss		1·46 gm.		1·72 gm.
Observed albumin loss		0·76 gm.		0·94 gm.
Amount of "new" albumin		0·70 gm.		0·78 gm.

Statistical analysis of some of the factors affecting the loss of total plasma protein and T1824 labelled protein during transfusion revealed qualitative differences in their effects on the plasma components. The results of the multiple regression analysis (Table 3) indicate that the loss of the T1824 labelled protein was directly related to the total excess tissue fluid retained and that the rise in venous pressure did not exert any additional effect. The total protein loss, however, was mainly influenced by a rise in venous pressure. The result of this dissociation in behaviour is interesting since the loss of T1824 labelled protein is an index of loss of plasma protein present in the circulation at the start of the experiment. The total circulating protein loss will depend on a balance between the loss of protein initially present in the circulation and the mobilization of new protein.

Table 4 shows the total amounts of protein and of T1824 lost from the plasma and recovered in the lymph in the various transfusion experiments. About 25 to 30 p.c. of the total dye lost from the plasma was recovered in the thoracic duct lymph during transfusion. This figure is in good agreement with the figure of about 30 p.c. of total excess tissue fluid filtered in the thoracic duct area.

Plasma protein loss in intact rabbits during transfusion. Twenty unanaesthetized rabbits in which the thoracic duct was not cannulated were transfused with varying amounts of Ringer-Locke solution for five hours. In some, there

TABLE 3.

Effect of total excess tissue fluid (ml./kg./5 hr.) and rise in inferior vena caval pressure (cm. H_2O) on plasma protein and T1824 loss, during intravenous Ringer-Locke infusion.

	No. of Experiments	Multiple regression equation	Mean protein or dye loss	First regression coefficient	Signif. of first regression coefficient	Second regression coefficient	Signif. of second regression coefficient
Total protein loss from plasma	8	P.L.=25—0·052 (T.E.T.F.—98)+9·62 (V.P.R.—2·0)	25± 1·6	0·052± 0·031	Not signif.	9·62± 2·34	Signif. p=0·001
T1824 loss from plasma	6	D.L.=50+0·116 (T.E.T.F.—88)+0·59 (V.P.R.—1·6)	50± 0·9	0·116± 0·036	Signif. p=0·05	0·59± 1·2	Not signif.

P.L. is protein loss as p.c. of amount circulating at start of experiment.
D.L. is T1824 loss as p.c. of amount circulating at start of experiment.
T.E.T.F. is total excess tissue fluid (ml./kg./5 hr.).
V.P.R. is average rise in venous pressure cm. H_2O.

was a significant loss of total circulating plasma protein. A multiple regression equation was derived from the data, relating protein loss to total excess tissue fluid retained and rise in aural venous pressure. The latter is known to reflect changes in central venous pressure during transfusion (Korner, 1953).

$$P.L. = 9 \cdot 1 + 0 \cdot 017\ (TETF - 123) + 1 \cdot 42\ (VPR - 9 \cdot 0)$$

where P.L. is the protein loss (p.c. of initial)
TETF is total excess tissue fluid (ml./kg./5 hr.)
VPR is venous pressure rise in cm. H_2O
The first regression coefficient is not significant ($p = 0 \cdot 6$); the second is significant ($p < 0 \cdot 001$).

The results indicate that the protein loss was mainly determined by the rise in venous pressure and was independent of the total amount of fluid retained. The protein loss in these intact rabbits was thus affected by similar factors to the loss of total protein in those experiments in which the lymph was collected.

TABLE 4.

Amount of protein and dye lost from plasma and recovered in lymph during I.V. infusion of Ringer-Locke.

	Protein lost from plasma mg./kg.	Protein recovered in lymph mg./kg.	Dye lost from plasma p.c. initial	Dye recovered in lymph p.c. initial
No. of experiments	9	9	6	6
Mean	607	795	50	14
S.E.	19·2	36·7	3·3	3·8

The effect of absorption of food and various fluids on the flow and protein content of thoracic duct lymph.

Effect of absorption of food. The experiments were carried out on two groups of cats. The first group of 23 was starved of all food for 24 hours preceding the experiment but were allowed water *ad lib.* The second group of 43 was given meat and milk on the afternoon before the experiment and again on the following morning. The thoracic duct was cannulated about 3 to 4 hours after the last feed and lymph collected for a period of 2 hours.

The spontaneous lymph flow was on the average 33 p.c. higher in the fed than in the post absorptive animals. The mean lymph flows were $2 \cdot 48 \pm 0 \cdot 16$ ml./kg./hr. in the fed group and $1 \cdot 86 \pm 0 \cdot 16$ ml./kg./hr. in the post absorptive group. Despite the overlap in lymph flow values between the two groups, this difference was statistically significant ($p = 0 \cdot 01$).

An analysis of some of the factors influencing the thoracic duct protein composition revealed certain qualitative differences between the two groups. In the case of the post absorptive animals it was found that the thoracic duct

protein concentration was directly related to the plasma protein concentration, but varied inversely with the rate of lymph flow. The results are summarized in the following multiple regression equation:

$$T.D.P.C.=4\cdot67+0\cdot656\ (P.P.C.-7\cdot02)-0\cdot428\ (L.F.-1\cdot86)$$

where T.D.P.C. is thoracic duct protein concentration gm. p.c.
P.P.C. is plasma protein concentration gm. p.c.
L.F. is lymph flow as ml./kg./hr.
Both regression coefficients are statistically significant ($p<0\cdot001$ and $0\cdot01$ respectively).

In the case of the fed cats it was found that the thoracic duct protein concentration was again directly related to the plasma protein concentration, but was independent of the rate of lymph flow. This is summarized in the following equation:

$$T.D.P.C.=4\cdot15+0\cdot772\ (P.P.C.-6\cdot55)-0\cdot082\ (L.F.-2\cdot48)$$

where the same symbols are used as in the previous equation,
The first regression coefficient is statistically significant ($p<0\cdot001$)
The second regression coefficient is not statistically significant ($p=0\cdot3$).

Fig. 6 illustrates in graphic form the qualitative differences in factors influencing the thoracic duct protein concentration in the two groups of cats.

Effect of gastric administration of water and Ringer-Locke solution. Seven cats were included in this study. Four animals were given Ringer-Locke solution by stomach tube at the rate of 40 ml./kg./hr., whilst three were given water at a similar rate. Fig. 7 shows the lymph flow patterns in typical experiments. During gastric administration of water, the lymph flow rose rapidly and reached a maximum in the second and third hour of infusion. Cessation of infusion was characterized by an abrupt fall in the rate of lymph flow, which returned to the previous resting value one hour after stopping the infusion. In contrast to the above, gastric administration of Ringer-Locke solution was accompanied by a more gradual change in the rate of lymph flow. The lymph flow rose during the third hour of the infusion and continued to increase for two hours after stopping the administration of fluid. Flow rates were still often above resting levels 5 hours after the infusion.

The influence of changes in plasma protein concentration and rate of lymph flow on the thoracic duct total protein and albumin concentrations was studied in the course of these experiments. The results have been summarized in a series of multiple regression equations and are given in Table 5. These indicate that during water absorption from the gut, the thoracic duct total protein and albumin concentrations were not related to the corresponding plasma levels, but varied inversely with the rate of lymph flow. During absorption of Ringer-Locke solution the lymph protein concentrations varied directly with the plasma

TABLE 5.

Regression of plasma protein and albumin concentration and rate of lymph flow on thoracic duct lymph protein and albumin concentration, during gastric administration of water and Ringer-Locke.

Experiment	No. of results	Multiple regression equation	First regression coefficient	Signif. of first regression coefficient	Second regression coefficient	Signif. of second regression coefficient
Water *per os*	10	T.D.P.C.=2·2+0·026 (P.P.C.—6·3)—0·235 (L.F.—5·9)	0·026± 0·2	Not signif.	0·235± 0·10	Signif. p=0·04
Water *per os*	10	T.D.A.C.=1·6+1·200 (P.A.C.—4·1)—0·167 (L.F.—5·9)	1·3± 1·0	Not signif.	0·167± 0·06	Signif. p=0·03
Ringer-Locke *per os*	18	T.D.P.C.=3·1+1·271 (P.P.C.—6·2)—0·341 (L.F.—4·8)	1·271± 0·56	Signif. p=0·03	0·341± 0·099	Signif. p=0·01
Ringer-Locke *per os*	18	T.D.A.C.=1·7+0·652 (P.A.C.—2·8)—0·189 (L.F.—4·8)	0·65± 0·29	Signif. p=0·05	0·189± 0·059	Signif. p=0·01

Same symbols as in Table 1.

levels and also inversely with the rate of lymph flow (Fig. 10). As will be seen later, these differences probably depend on both the rate and pathway of absorption of the two fluids.

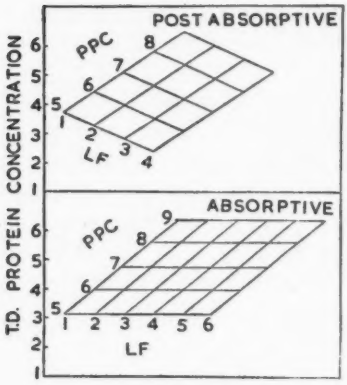

Fig. 6.

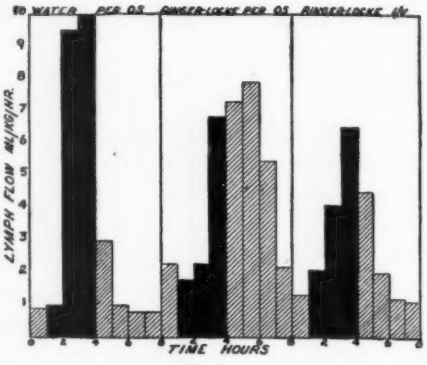

Fig. 7.

Fig. 6. Nomogram showing effect of plasma protein concentration (gm. p.c.) and rate of lymph flow (ml./kg./hr.) on thoracic duct protein concentration (gm. p.c.) in animals absorbing food and in post absorptive cats. The ordinate of the diagram is thoracic duct protein concentration. The lymph protein concentration for any given plasma protein concentration (P.P.C.) and rate of lymph flow (L.F.) may be obtained by reading off the appropriate P.P.C. and L.F. values. The corresponding lymph protein concentration will be the value of the ordinate corresponding to the point of intersection of the P.P.C. and L.F. values.

Fig. 7. Lymph flow patterns during and after intragastric administration of water and Ringer-Locke solution and intravenous administration of Ringer-Locke solution. All fluids given at the rate of 40 ml./kg./hr.

■ period of fluid administration.
▩ pre and post infusion period.

Electrophoretic studies on plasma and lymph.

Figs. 8A and 8B show typical plasma and lymph protein patterns in resting experiments. It is obvious from inspection that the A/G ratio was greater in lymph than in plasma in agreement with the results of chemical fractionation. All protein fractions present in the plasma were also represented in the lymph. During intravenous transfusions of Ringer-Locke the A/G ratio in the lymph was practically the same as in resting lymph. The most interesting patterns were those following oral administration of water (Figs. 8C and 8D). At the point "H" of the diagram there was present in both plasma and lymph a considerable quantity of free haemoglobin. The amount of water given to the cats was very large (120 ml./kg./3 hr.) and always produced quite a noticeable degree

of intravascular haemolysis. Free haemoglobin appeared in the lymph in recognisable amounts about 1 to 2 hours after the plasma showed signs of haemolysis.

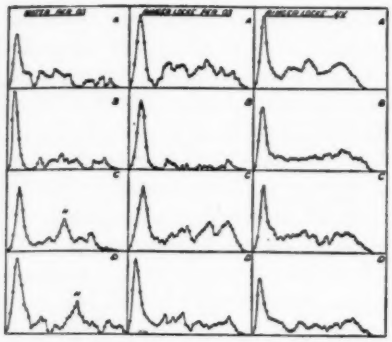

Fig. 8. Electrophoretic patterns in plasma and lymph before and during administration of water *per os*, Ringer-Locke *per os*, and Ringer-Locke intravenously.

A=Pre-infusion plasma.
B=Pre-infusion lymph.
C=Plasma during infusion.
D=Lymph during infusion.

DISCUSSION.

General factors affecting lymph flow and protein composition.

It is important to consider how far it is possible from the collection of lymph to gain an idea of fluid and protein exchanges between capillaries and the extravascular fluid. It is obvious that the lymph flow over a prolonged period of time affords a measure between the imbalance of the filtering and absorbing forces governing fluid exchange. The protein composition of lymph also represents the interaction of many factors. These include (i) the protein composition of the capillary filtrate, (ii) the degree of concentration of this filtrate during reabsorption of water, (iii) the extent and composition of the extravascular protein pool, and (iv) the absorption of non-protein fluid from the gut. The protein concentration of tissue fluid is probably not uniform throughout the interstitial space. The diffusion coefficients of the plasma proteins are such that relatively large concentration gradients are possible between tissue fluid undergoing absorption at the venular end of the capillaries and tissue fluid composed of freshly formed filtrate (Pappenheimer and Soto-Rivera, 1948). This interaction of a large number of variates in lymph, renders interpretation of lymphatic experiments difficult, except under circumscribed conditions.

Factors influencing the flow and composition of lymph during intravenous infusions.

With graded massive infusions changes in lymph flow and lymph protein composition will largely reflect changes in capillary filtration. In the transfusion experiments there was a raised venous and probably capillary hydrostatic pressure. This would enhance the filtration of fluid and diminish reabsorption. The effect was most striking with the largest net infusions (180 to 200 ml./kg./5 hr.), and under these conditions bulk reabsorption of fluid from the intravascular space back into the capillaries was probably slight. The greater

the total excess tissue fluid present the more will the lymph resemble the capillary filtrate and the more will factors leading to enhanced filtration be reflected in an increase in lymph flow.

From the proportion of excess lymph production to total excess tissue fluid the conclusion was reached that approximately 30 p.c. of the excess tissue fluid was filtered into the thoracic duct bed. Approximately 30 p.c. of the T1824 labelled protein lost from the plasma was recovered in the thoracic duct lymph. It follows that increased fluid filtration during transfusion is accompanied by a proportional increase in protein filtration. The fractional composition of the lymph did not alter during rapid capillary filtration, as evidenced by the fact that the average A/G ratios were the same at rest and during transfusion. The capillary membrane presents normally greater resistance to the passage of globulin molecules than to albumin molecules (Pappenheimer, 1953). Our experiments suggest that at high filtration rates this difference in permeability is still maintained to approximately the same extent, although the total quantity of bulk transfer is increased.

Analysis of factors influencing lymph protein composition during steady state conditions with transfusion, showed that the thoracic duct protein concentration varied directly with the plasma protein concentration and was independent of the rate of lymph flow (Table 2). The lack of significance of the lymph flow factor was surprising, as it was thought that with large flows there might be a disproportionate dilution of the extravascular protein component of the thoracic duct lymph. This might be expected, even though increased filtration of fluid was accompanied by proportionate protein filtration. The results, however, clearly indicate that this was not the case. The importance of plasma protein concentration in determining lymph protein concentration shows that during transfusions there is a steady plasma-lymph equilibrium.

With very large net infusions (150 to 180 ml./kg./5 hr.) the lymph protein concentration dropped very rapidly to about 0·8 to 0·9 gm. p.c. From the results of other authors using different techniques, estimates of the protein composition of capillary filtrate have been reported from 0·3 to 0·8 gm. p.c. in different areas (Landis and Gibbon, 1933; Landis, Krogh and Turner, 1932; Landis, 1934; Pappenheimer, Renkin and Borero, 1951). Assuming that the protein content of the capillary filtrate in the thoracic duct bed is about 0·5 gm. p.c., it is possible to get an approximate idea of the amount of extravascular protein compared to plasma filtrate in lymph during various degrees of transfusion. It may be seen in Figs. 9A and 9B that with small net infusions (40 ml./kg./5 hr.) at the end of 5 hours 65 p.c. of the lymph protein arises from the extravascular protein pool and 35 p.c. represents plasma filtrate. With a net infusion of 180 ml./kg./5 hr. (Figs. 9C and 9D), most of the lymph protein after 2 hours represents plasma filtrate and the extravascular protein pool has, to a large extent, become washed out.

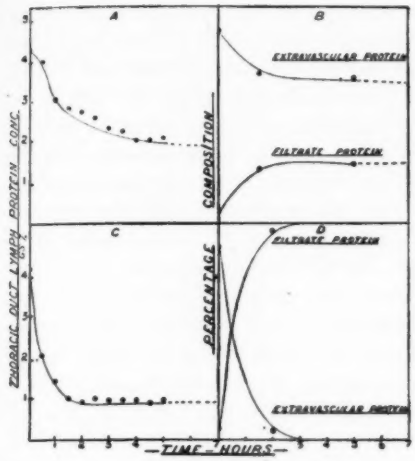

Fig. 9.

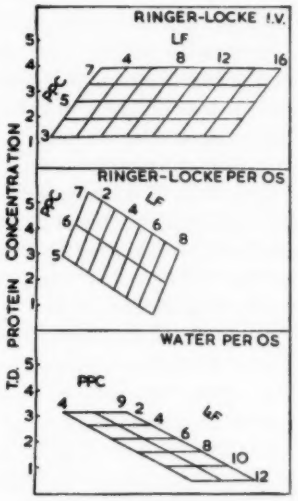

Fig. 10.

Fig. 9. Effect of different degrees of transfusion on thoracic duct protein composition.

A and B=Net infusion of 40 ml./kg./5 hr.
C and D=Net infusion of 180 ml./kg./5 hr.

A and C show lymph protein concentration (gm. p.c.), and B and D show the approximate contributions of extravascular protein and capillary filtrate to the composition of the lymph.

Fig. 10. Nomogram showing effect of plasma protein concentration (P.P.C.) and rate of lymph flow (L.F.) on thoracic duct protein concentration (gm. p.c.), during administration of water *per os*, Ringer-Locke *per os*, and Ringer-Locke intravenously. Lymph protein concentration may be read off the diagram by the method described in the legend for Fig. 6.

Absorption of fluid and food from the intestines.

The absorption experiments fall into two categories. In one group large quantities of simple solutions were administered to the animals and the changes in the lymph were probably mainly influenced by the rate and the pathway of absorption. In the other group complex foods were absorbed. In these experiments the underlying changes are considerably more complex in that digestion will be accompanied by an intestinal hyperaemia. The changes in the lymph during food absorption will thus be affected both by the direct absorption of the end products of digestion and by the increased filtration resulting from the intestinal hyperaemia.

Comparison of the lymph flow patterns during absorption of water and Ringer-Locke solution showed that water increased the flow of lymph much more rapidly than Ringer-Locke solution. The results of the analysis of factors influencing thoracic duct protein composition during the absorption of the above

fluids are shown in Fig. 10. During water absorption the thoracic duct protein concentration varied inversely with the rate of lymph flow and was independent of the plasma protein concentration. The most likely interpretation of these facts is that water is rapidly absorbed by the intestinal epithelium and dilutes the extravascular protein pool in the villi to a sufficient extent to disturb the normal plasma extravascular protein equilibrium completely. Most of the absorbed fluid is transferred into the lymphatics, though, from inspection of the electrophoretic patterns in Fig. 8, some of the water finds its way into the blood capillaries and is eventually filtered from the blood stream. From the results of the flow patterns of Ringer-Locke by mouth, it seems likely that some of it is absorbed directly into the blood stream and filtered secondarily. The fact that the thoracic duct protein concentration was still directly related to the plasma protein concentration shows that absorption was sufficiently slow not to disturb the normal plasma-lymph equilibrium. There was, in addition, an inverse relationship between thoracic duct protein concentration and rate of lymph flow. This implies some disproportionate dilution of the extravascular protein in the intestinal villi and could be due either to direct transference of the Ringer-Locke into the lymph capillaries or due to secondary filtration.

In the food absorption experiments our results show definite differences in behaviour between the absorptive and post-absorptive cats, and these are summarized in Fig. 6. In the post-absorptive cats a rise in the capillary filtration pressure will lead to increased filtration of fluid, a rise in tissue pressure, and, eventually, an increase in lymph flow. The increased filtration of fluid will dilute the extravascular protein pool and reduce the lymph protein level. The changes in the lymph during digestion are far more difficult to interpret as in these experiments the protein concentration was independent of the lymph flow. It is likely that digestion is associated with an intestinal hyperaemia and a resultant increase in capillary filtration area. Korner and Courtice (1954) have recently shown that in a constricted vascular bed the size of the effective extravascular protein pool is smaller than in a normal vascular bed. It is possible that in a dilated vascular bed the effective extravascular protein pool in association with the capillaries is increased. Thus a given volume of filtrate would mix with a larger amount of interstitial protein in the fed cats than in the post-absorptive cats, and any tendency for the lymph protein concentration to drop would be masked. It must be emphasized that the magnitude of the extravascular protein pool in relation to the amount of protein filtered masks the effect of possible slight alterations in capillary permeability during digestion.

Mobilization of "new" protein during intravenous transfusion.

During the lymphatic transfusion experiments, about half the loss of protein initially present in the circulation had been made good by the re-entry of new protein. The chief factor apparently preventing the entry of new protein was

a rise in venous pressure. In the intact unanaesthetized rabbits there was also a loss of total circulating protein determined by a rise in central venous pressure. The loss of protein was, however, much slighter than in the lymphatic experiments. With a rise of venous pressure in the ear vein of 9 cm. (corresponding to a central venous pressure of 2 to 3 cm.) the protein loss was only 9 p.c. compared to a 20 p.c. loss in the lymphatic experiments. This difference emphasizes the great importance of the thoracic duct lymph return in maintaining the total amount of circulating plasma protein during enhanced capillary filtration. It seems possible that in the rabbits the rise of central venous pressure might prevent the entry of thoracic duct lymph at the venous confluence by mechanical damming back of lymph. In the cat experiments with prolonged thoracic duct drainage, the entry of new protein is more difficult to explain. By analogy with the rabbit experiments it is possible that the rise of venous pressure led to damming back of lymph from some other lymphatic. The only duct where this could be accomplished under anaesthesia would be the right lymph duct. Even then one would have to postulate more rapid flow rates than are known to occur (Courtice, Harding and Steinbeck, 1953). An alternative explanation may be that new protein is formed in response to excessive protein loss following prolonged lymph drainage. Such protein would probably be formed in the liver and could enter the circulation directly. From the results of Forker, Chaikoff and Reinhardt (1952), on prolonged lymph drainage in rats, it is possible that such a mechanism may operate.

SUMMARY.

In cats actively absorbing food the lymph flow was 30 p.c. higher than in post-absorptive resting cats. The thoracic duct protein concentration varied directly with the plasma protein concentration and was independent of the rate of lymph flow in cats absorbing food; it varied directly with plasma protein concentration and inversely as the lymph flow in post-absorptive cats.

Oral administration of water and Ringer-Locke at the rate of 40 ml./kg./hr. produced differences in lymph flow patterns. Water was absorbed for the most part into the extravascular space of the intestinal villi and produced a dilution of the lymph. Some water was directly absorbed into the blood stream, as judged by the production of intravascular haemolysis. Ringer-Locke solution given orally was much more slowly absorbed, a large part directly into the blood stream and some directly into the extravascular space of intestinal villi.

Massive intravenous infusions of Ringer-Locke greatly increased the lymph flow and accelerated the attainment of plasma-lymph protein equilibrium. By the use of the massive infusion technique the size of the capillary bed drained by the thoracic duct was estimated at 30 p.c. of the total body filtration area.

The protein composition of thoracic duct lymph during steady state conditions varied directly as the plasma protein concentration, and was independent

of the rate of lymph flow. It was found that the increased filtration of fluid was accompanied by a proportional increase in filtration of protein. The A/G ratios of plasma and lymph showed a significant difference. The A/G ratio of the lymph remained unaltered during transfusion.

There was evidence that new protein entered the circulation to make good some of the loss of protein resulting from lymphatic drainage during massive intravenous infusion. The greatest total protein loss occurred with the largest rises in venous pressure. The possible mechanisms of entry of new protein into the circulation and the rôle of the venous pressure changes have been discussed.

Electrophoretic patterns of plasma and lymph during the various procedures have been presented.

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THE EFFECTS OF ACUTE ANOXIA AND NORADRENALINE VASOCONSTRICTION ON LYMPH FLOW AND PROTEIN DYNAMICS FOLLOWING TRANSFUSIONS OF RINGER-LOCKE SOLUTION[1]

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There is still disagreement in the literature regarding the effects of acute anoxia on capillary permeability to protein. On the basis of increases in lymph flow and lymph protein output from the right lymph duct, it has been thought that acute anoxia increased the permeability of the lung capillaries to both fluid and protein (Warren and Drinker, 1942; Warren, Peterson and Drinker, 1942; Drinker, 1945). Similar increases in lymph flow have been obtained from the thoracic duct (Beznak and Liljestrand, 1949). Landis (1927) demonstrated changes in the capillaries of the frog's mesentery with extreme grades of anoxia. McMichael and Morris (1936) found no increase in the rate of swelling of the human forearm after breathing 9·5 p.c. O_2 in N_2, suggesting that the permeability of the arm capillaries to fluid was not altered by anoxia. The studies of Stead and Warren (1944), Henry, Goodman and Meehan (1947) also failed to show changes in the permeability of fluid and protein of the capillaries of the extremities, except with very severe degrees of anoxia. Courtice and Korner (1952) showed that there was probably no differential increase in pulmonary capillary permeability in rabbits during prolonged anoxia. In a recent paper, Korner, Morris and Courtice (1954) pointed out that it was difficult to demonstrate any changes in capillary permeability from studies of resting lymph flow and lymph protein composition. When changes in capillary permeability are very gross, such as after thermal burns (c.f. Glenn, Peterson and Drinker, 1942; Courtice, 1946), the protein composition of the lymph will approach that of plasma, the changes in permeability then being obvious. Normally, or with slight changes in capillary permeability, the volume and composition of lymph will not reflect directly any alterations in volume and protein content of capillary filtrate, since the large extravascular protein pool will tend to mask such changes. Korner *et al.* (1954) showed that during prolonged intravenous infusions of Ringer-Locke solutions, it was possible to evaluate changes in lymph in terms of alterations in capillary filtration.

[1]This work was carried out with the aid of a grant from the National Health and Medical Research Council, Canberra.

Accordingly, in the present experiments thoracic duct lymph flow and protein composition have been studied during intravenous infusions of Ringer-Locke solution in three groups of cats. The first group of cats was transfused whilst breathing room air, the second whilst breathing 10 p.c. oxygen in nitrogen; the third group was transfused whilst receiving noradrenaline at the rate of 0·3 μg./kg./minute. Since it became apparent that anoxia in the present experiments produced systemic vasoconstriction, it was thought desirable to compare its effects with the known vasoconstrictor l-noradrenaline.

METHODS.

The experiments were carried out on 25 cats lightly anaesthetized with intravenous nembutal.

The method of thoracic duct cannulation, as well as all analytical procedures, was the same as described by Korner, Morris and Courtice (1954). All cats were infused with Ringer-Locke for a period of five hours.

Low oxygen mixtures were administered from gas cylinders as described by Courtice and Korner (1952).

The noradrenaline was infused at the rate of 0·3 μg./kg./minute (Korner, 1953).

RESULTS.

Lymph flow, venous and arterial pressure changes during intravenous Ringer-Locke transfusion.

The changes in rate of lymph flow and venous and arterial pressures with varying infusions are shown in Fig. 1. It may be seen that there was no significant difference in lymph flow patterns in cats transfused while breathing air,

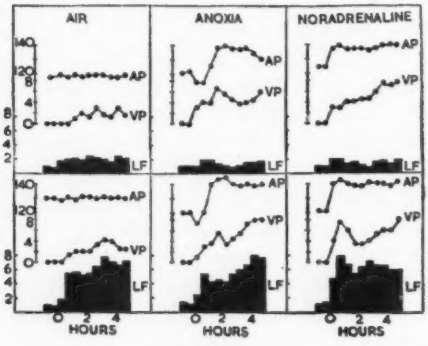

Fig. 1.

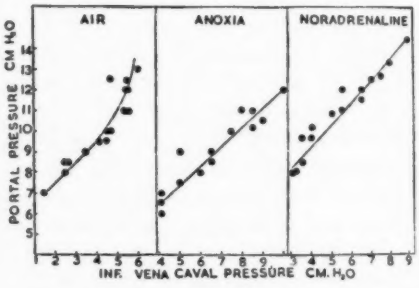

Fig. 2.

Fig. 1. Variation in lymph flow (L.F.=ml./kg./½ hr.), inferior vena caval pressure (V.P.=cm. H_2O) and arterial pressure (A.P.=mm. Hg.) in cats transfused from 0-5 hours whilst breathing air, 10 p.c. O_2, or receiving noradrenaline.

Upper graphs=net infusion of 30 ml./kg./5 hr.
Lower graphs=net infusion of 180 ml./kg./5 hr.

Fig. 2. Simultaneous measurement of lower inferior vena caval pressure and portal pressure during transfusion of Ringer-Locke in the three groups of cats.

TABLE 1.

Relation between thoracic duct lymph volume (ml./kg./5 hr.) and total excess tissue fluid (ml./kg./5 hr.) retained by animal, in cats transfused whilst breathing air, 10 p.c. O_2 in N_2, or receiving noradrenaline (0·3 µg/kg./minute).

Group	No. of experiments	Regression equation	Regression coefficient	Significance of regression coefficient	Lymph volume for a total excess tissue fluid of 100 ml./kg./5 hr.
Air	9	L.V.=32+0·240 (T.E.T.F.—103)	0·240±0·056	p=0·001	31 ml./kg./5 hr.
10 p.c. O_2	8	L.V.=26+0·205 (T.E.T.F.— 88)	0·205±0·026	p<0·001	28 ml./kg./5 hr.
Noradrenaline	5	L.V.=31+0·287 (T.E.T.F.—104)	0·287±0·019	p<0·001	30 ml./kg./5 hr.

L.V. is lymph volume (ml./kg./5 hr.).
T.E.T.F. is total excess tissue fluid retained (ml./kg./5 hr.).

10 p.c. O_2, or while receiving noradrenaline. In the latter two groups, however, there was a rise in mean femoral arterial pressure of about 15-20 mm. Hg; the rise in lower inferior cava pressure was greater in the cats receiving 10 p.c. O_2 in N_2, or noradrenaline, than in those breathing air. There was a linear relationship between the lymph volume produced during 5 hours transfusion (ml./kg./5 hr.) and the total excess tissue fluid retained by the animals (i.e., volume of Ringer-Locke infused minus urine produced minus excess fluid in plasma).

The results in the three groups are shown in Table 1. In the cats breathing air, unit change in the total excess tissue fluid retained resulted in a 24 p.c. rise in lymph production. The anoxic cats showed a 21 p.c. rise in lymph production for a unit change in total excess tissue fluid, whilst the noradrenaline group showed a 29 p.c. increase in lymph flow. The anoxia and noradrenaline groups thus show similar increases in lymph flow for a given net infusion as the group breathing air. In anoxia there was a small transient increase in respiratory rate and minute volume, which soon returned to normal. In cats lightly anaesthetized with nembutal there was no systematic difference in the respiratory pattern in all three groups.

TABLE 2.

Effect of transfusion on lower inferior vena caval pressure, plasma and lymph protein concentrations, and plasma-lymph osmotic pressure gradient in the three groups of cats.

Group	No. of experiments	Mean of average rise in inferior vena cava pressure cm. H_2O	Average plasma protein conc. gm. p.c.	Average lymph protein conc. gm. p.c.	Gradient in colloid O.P. between plasma and lymph cm. H_2O
Air	9	1·84±0·29	4·91±0·14	2·21±0·12	11
10 p.c. O_2	8	4·1 ±0·46	5·30±0·31	2·20±0·22	12
Noradrenaline	5	4·6 ±0·45	4·80±0·06	1·5 ±0·24	13

The haemodynamic alterations, however, are different in the three groups. Table 2 shows that there was a greater rise in inferior vena caval pressure in the anoxia and noradrenaline groups than in the group breathing air. The osmotic pressure gradient between plasma and lymph, as estimated from the change in protein concentration (Wells, Youmans and Miller, 1932), was similar in all three groups.

Fig. 2 shows changes in venous pressure measured simultaneously in the lower inferior vena cava and portal vein during transfusion of Ringer-Locke in typical experiments on cats breathing air, 10 p.c. O_2, or receiving noradrenaline. In all three groups the relation between the two measurements was similar, though the portal pressure was always at a higher absolute level. An estimate of rise in lower inferior caval pressure during transfusion will thus give a good estimate of changes in portal venous pressure.

TABLE 3.

Effect of plasma protein concentration and rate of lymph flow on thoracic duct protein concentration in all three groups.

Group	No. of results	Multiple regression equation	First regression coefficient	Signif. of first regression coefficient	Second regression coefficient	Signif. of second regression coefficient
Air	32	T.D.P.C.=2·22+0·557 (P.P.C.—4·90)—0·03 (L.F.—8·7)	0·557± 0·046	$p<0·001$	0·031± 0·022	Not signif.
10 p.c. O_2	20	T.D.P.C.=2·20+0·256 (P.P.C.—5·37)—0·081 (L.F.—6·9)	0·256± 0·104	$p=0·02$	0·081± 0·022	$p=0·001$
Noradrenaline	15	T.D.P.C.=1·5 +0·256 (P.P.C.—4·80)—0·154 (L.F.—6·6)	0·256± 0·366	Not signif.	0·154± 0·047	$p=0·01$

T.D.P.C.=Thoracic duct protein concentration gm. p.c.
P.P.C.=Plasma protein concentration gm. p.c.
L.F.=Lymph flow (ml./kg./hr.).

The rise in mean femoral arterial pressure is suggestive of systemic vasoconstriction, from what is known of cardiac output changes in animals breathing 10 p.c. O_2 and receiving noradrenaline (Doi, 1921; Warren, Peterson and Drinker, 1942; Courtice and Korner, 1952; Lewis and Gorlin, 1952; Korner, 1954). It follows then that in the anoxia and noradrenaline groups the greater rise in venous pressure allows the same amount of fluid filtration as occurs with air through a constricted vascular bed.

Factors influencing protein composition of thoracic duct lymph.

The relations of plasma protein concentration and rate of lymph flow on thoracic duct protein concentration in all three groups were investigated during steady state conditions (i.e., after about 2½ hours transfusion). The results have been summarized in Table 3. In the group breathing air the thoracic duct protein concentration varied directly with the plasma protein concentration and was independent of the rate of lymph flow. In the anoxic cats the thoracic duct

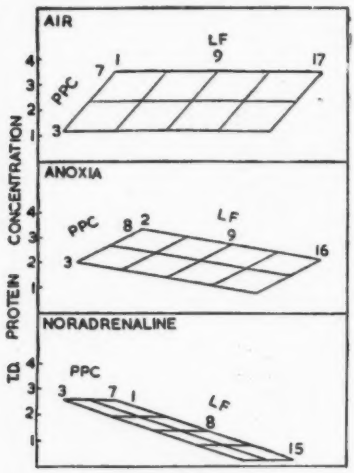

Fig. 3.

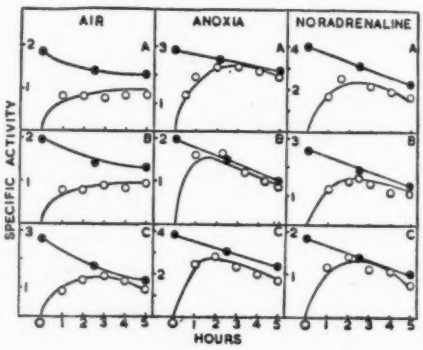

Fig. 4.

Fig. 3. Nomogram showing relation of plasma protein concentration (P.P.C.=gm. p.c.) and lymph flow (L.F.=ml./kg./hr.) on thoracic duct protein concentration (gm. p.c.) in the three groups of cats. The thoracic duct protein concentration can be read off for any particular P.P.C. and L.F. values by reading off the latter along the appropriate P.P.C. and L.F. lines. The thoracic duct concentration for those values will be the ordinate value corresponding to the point of intersection of the P.P.C. and L.F. lines.

Fig. 4. Specific activities (T1824 concentration/total protein concentration) in plasma and lymph in the three groups of cats.

A=no transfusion.
B=net infusion of 40 ml./kg./5 hr.
C=net infusion of 120 ml./kg./5 hr.
●=specific activity of plasma.
O=specific activity of lymph.

protein concentration varied directly with the plasma protein concentration and inversely as the rate of lymph flow. In the noradrenaline group the thoracic duct protein concentration was independent of the plasma protein concentration but varied inversely with the rate of lymph flow. These regression equations are illustrated graphically in the nomogram in Fig. 3. It may be seen that unit change in plasma protein concentration produces a significantly smaller change in thoracic duct protein composition in the anoxic group than in the cats breathing air. In the noradrenaline cats unit change in lymph flow produced a slightly greater dilution of the thoracic duct lymph than in the anoxic group. The significance of these findings will be considered below.

Plasma-lymph exchange dynamics.

In Fig. 4 the specific activities (T1824 concentration/total protein concentration) in plasma and lymph have been plotted in experiments with different net infusions. It is apparent that, in the group of cats breathing air, equilibrium was not attained during the five-hour period with low net infusions; with large transfusions equilibrium was reached 2 to 3 hours after beginning the infusion. In the anoxic and noradrenaline group equilibrium was reached even with very low net infusions after 2 to 3 hours.

TABLE 4.

Loss of total protein and T1824 labelled protein from plasma and their recovery in thoracic duct lymph following five hours intravenous infusion.

		Loss from plasma			Recovery in lymph	
Group	No. of experiments	Total protein loss mg./kg./5 hr.	Total protein loss p.c. of initial	T1824 loss p.c. of initial	Total protein recovered mg./kg./5 hr.	T1824 recovered p.c. of initial
Air	9	610±21	25±3·5	50±3·1	795±14	14·6±3·8
10 p.c. O_2	8	586±22	23±3·7	55±3·3	582±10	12·8±3·9
Noradrenaline	5	500±10	24±1·1	59±1·8	374±17	9·8±1·7

The results of total protein and T1824 loss from the plasma and total protein and T1824 recovery in lymph are summarized in Table 4. The total protein and T1824 loss from the plasma was very similar in all three groups; about 25 p.c. of the total circulating protein was lost in the course of 5 hours lymphatic drainage, and a little over 50 p.c. of the circulating T1824. The discrepancy between protein and dye loss has been fully discussed by Korner *et al.* (1954). The amount of total protein recovered in the lymph was somewhat less in the anoxia and noradrenaline groups than in the cats transfused while breathing air. The amount of T1824 recovered in the lymph, expressed as a percentage of the amount circulating at the beginning of the experiment, was not significantly different in any of the groups.

In order to gain some idea of the relation between protein and fluid filtration from the plasma, the amount of T1824 recovered in the lymph in various

half-hour periods was expressed as a percentage of the amount of T1824 circulating in the plasma at that time. The relation between rate of lymph flow and percentage of T1824 recovered in the lymph was examined by deriving two regression equations. Points were only included after 2 hours transfusion when a reasonable degree of plasma-lymph exchange had taken place and enhanced filtration, due to transfusion, had become well established. In the air experiments the following equation was derived:

$$A.D.F.=2{\cdot}02+0{\cdot}386\ (L.F.-3{\cdot}7)$$

where A.D.F. is amount of lymph T1824/½ hr. as a percentage of average T1824 level in the plasma

L.F. is lymph flow (ml./kg./½ hr.)

The regression coefficient is statistically significant ($p<0{\cdot}001$).

In the anoxic animals the following equation was derived:

$$A.D.F.=2{\cdot}20+0{\cdot}302\ (L.F.-3{\cdot}7)$$

where the same symbols are used as in the above equation

The regression coefficient is statistically significant ($p=0{\cdot}01$).

In the air experiments the average amount of dye filtered per half hour was 2·02±0·22 p.c. of the amount circulating. Unit rise in the rate of lymph flow increased the amount of dye recovered in the lymph by about 0·4 p.c. The amount of dye recovered in the lymph varied directly as the lymph flow. Exactly the same conclusions apply to the anoxia. With noradrenaline no such definite relation could be established, but the mean figures were 1·7±0·8 p.c. T1824 recovered in lymph for a lymph flow of 3·6 ml./kg./½ hr., which do not differ significantly from the air and anoxia results. The importance of these dye results in interpreting the changes in lymph protein composition will be considered in the discussion.

DISCUSSION.

Lymph flow during intravenous infusion of Ringer-Locke solution.

The total lymph volume collected in the five-hour experiments was not significantly different in any of the groups for a given net infusion level, and unit change in net infusion resulted in a similar increment of lymph flow. This result was obtained despite the striking differences in the circulatory findings. The circulatory changes were very similar in the anoxia and noradrenaline groups, though they were slightly more pronounced in the latter. The findings of systemic hypertension and elevated venous pressures are very suggestive of systemic vasoconstriction, with elevation of venous pressure resulting from vasomotor redistribution of blood (Korner, 1953). This is especially likely from what is known about the effects of anoxia and noradrenaline on the cardiac output. With the latter drug Goldenberg, Pines, Baldwin, Greene and Rob (1948) found no change in cardiac output in man, and Korner (1953) found no signi-

ficant change in cardiac output in unanaesthetized rabbits. With anoxia of much shorter duration than used in the present experiments, slight rises of cardiac output have been reported in man (Fishman, McClement, Himelstein and Cournand, 1952). In the anaesthetized cat with degrees of anoxia comparable to those of the present experiments, Doi (1921) found no change, or a slight fall in cardiac output, whilst in the dog Lewis and Gorlin (1952) reported little change. In unanaesthetized rabbits, which are probably more affected by anoxia than other animals, a considerable fall in cardiac output has been found to occur (Courtice and Korner, 1952; Korner, 1954). In view of these findings it seems certain that there was systemic vasoconstriction in the anoxia and noradrenaline experiments. Systemic vasoconstriction *per se* would tend to lower the capillary pressure, but in the present experiments this effect would be counterbalanced by the greater rise in venous pressure. This would increase the capillary hydrostatic pressure and also diminish reabsorption of water from the venular ends of the capillaries. There was no evidence that under the experimental conditions there was an overall increased filtration of fluid in anoxia. Landis (1934, 1946) has pointed out that vasoconstrictor changes in a capillary bed can mask changes in permeability of individual capillaries; the analysis of the flow data above does not therefore exclude the possibility that some of the capillaries in the constricted vascular bed in anoxia may not be somewhat more permeable to fluid. However, as will be seen later, the present experiments suggest that there is not even a limited rise in permeability in the sense referred to above.

Protein changes in plasma and lymph.

The loss of total protein and T1824 labelled protein was similar in all three experimental groups, and the amounts of T1824 recovered in the thoracic duct lymph were also similar. This suggests that there is no gross change in capillary permeability in anoxia as postulated by Warren *et al.* (1942). A striking difference in these experiments was the much more rapid rate of equilibration of plasma and lymph specific activities, which occurred with low net infusions and even without transfusion in the noradrenaline and anoxia groups compared to the air group. Wasserman and Mayerson (1952) have suggested that two para-meters may affect the time of equilibrium of specific activity between plasma and lymph. These are, (1) the rate of plasma protein leakage, and (2) the amount of protein present in the interstitial fluid with which the leaked protein must mix. From the results of the present experiments the importance of both these para-meters may be assessed. Thus, with the air experiments, the average amount of T1824 recovered in the lymph was 2·02 p.c. of the circulating amount with an average lymph flow of 3·7 ml./kg./½ hr.; unit increment of lymph flow produced an increment of 0·4 p.c. in the amount of T1824 recovered. In anoxia the average amount of T1824 recovered in the lymph was 2·20 p.c. of the circulating amount with an average lymph flow of 3·7 ml./kg./½ hr.; unit

increment of lymph flow resulting in an increment of 0·3 p.c. in the amount of T1824 recovered. The amount of T1824 recovered in the lymph at different lymph flows will afford some measure of the percentage of plasma protein filtered from the blood capillaries. It is clear, therefore, from the above results that there is no greater rate of plasma protein leakage in the anoxia and noradrenaline experiments than in the air group. This may also be seen from the results of Table 4. We can conclude then that increased rate of protein leakage does not account for the more rapid equilibrium of specific activities in the anoxia and noradrenaline groups compared to air.

The analysis of factors influencing thoracic duct protein concentration is of particular value in assessing the magnitude of the second para-meter referred to by Wasserman and Mayerson. In Table 3 it was shown that the thoracic duct protein concentration was affected by qualitatively different factors in all the three experimental groups. Korner *et al.* (1954) have shown that with cats breathing air there is a proportional amount of fluid and protein filtered from the capillaries during transfusion, with no disproportionate dilution of the extravascular protein at different flow rates. A comparison of the partial regression coefficients expressing the regression of plasma protein concentration on thoracic duct protein concentration in the air and anoxia groups reveals differences between the two. Thus with air unit change in plasma protein concentration produced a change of 0·56 p.c. in thoracic duct protein concentration; in anoxia, however, a similar change in plasma protein concentration only resulted in a change of 0·26 p.c. The difference in the regression coefficients is 0·30±0·13 and is statistically significant ($p=0·02$). At high lymph flows there is a greater gradient between plasma and lymph with anoxia than with air. The fundamental implication of this finding is that there is greater dilution of the interstitial protein pool by the capillary filtrate. This could be due to either a lower protein concentration of the extravascular fluid, or to a larger volume of filtrate due to reduced reabsorption of water from the venular ends of the capillaries. The significance of the lymph flow factor is also of interest. As was shown in the T1824 experiments, the total amount of protein filtered from the capillaries was practically the same in all three groups. The significance of the lymph flow factor in the anoxia and noradrenaline experiments must mean that there is a disproportionate amount of dilution of the protein present in the insterstitial fluid. As the amount of lymph produced was the same, it follows that the amount of interstitial protein must have been reduced.

Thus the more rapid equilibrium with anoxia and noradrenaline is not attained because of a more rapid rate of leakage of plasma proteins, but because of a smaller effective extravascular space. A further point indicating this is a comparison of the lymph protein concentrations obtained with the largest transfusions in all three groups. With the largest net infusions lymph will most closely resemble capillary filtrate (Korner *et al.*, 1954). With net infusions of

200 ml./kg./5 hr. the lowest protein concentration obtained in lymph was 0·9 gm. p.c. with air, 0·6 gm. p.c. with anoxia, and 0·3 gm. p.c. with noradrenaline. The latter lymph probably had the smallest admixture of interstitial protein and thus resembles most closely the true capillary filtrate. It is also clear from the latter figures that there can be almost no change in capillary permeability to protein even in individual capillaries during anoxia. These figures are in agreement with the results of Landis, Krogh and Turner (1932), Landis, Jonas, Angevine and Erb (1932), and Pappenheimer, Renkin and Borero (1952) for the capillary filtrate of the human forearm and hind limb of the cat. It would appear that the visceral capillaries are not particularly more permeable to protein than those of other regions.

It remains to consider the possible anatomical basis of the reduction in effective extravascular space in the anoxia and noradrenaline experiments. The extravascular space is in intimate contact with the blood capillaries. Small lymph capillaries arise in it and join on with others from the small interstitial area surrounding individual capillaries to form the main lymphatic trunks. Capillary filtrate from an individual capillary thus only mixes with a relatively small interstitial fluid and protein component before entering the lymph. From the work of Chambers and Zweifach (1947) it would appear that, during systemic vasoconstriction, blood would flow mainly through the A-V capillary channels and less through the "true" capillaries. A greater number of arterioles per unit mass of tissue will probably be closed almost completely during increased vasomotor activity (Nicol, Girling, Jerrard, Claxton and Burton, 1952). In this way the capillary filtration area would be reduced during vasoconstriction. With a reduction of the area of filtration, the effective interstitial fluid pool with which the capillary filtrate must mix will be reduced by a corresponding amount. If then a normal amount of fluid filters through a reduced capillary area as under transfusion conditions, it will mix with a relatively smaller amount of extravascular protein, and in this way a more rapid equilibrium of specific activities between plasma and lymph will be reached. It should be noted that the only way in which our results can be explained adequately involves an anatomical reduction in the size of the capillary filtration area during vasoconstriction produced by noradrenaline and anoxia.

In conclusion, it may be said that anoxia of the degree used in these experiments probably does not lead to an increased capillary permeability, but by means of its vasomotor effects, produces alterations in the plasma-lymph exchange dynamics.

SUMMARY.

There was no difference in the amount of lymph produced for a given net infusion of Ringer-Locke solution in cats transfused whilst breathing air, 10 p.c. O_2 in nitrogen, or receiving noradrenaline at the rate of 0·3 μg./kg./minute.

The groups breathing 10 p.c. O_2 in N_2 and receiving noradrenaline showed evidence of systemic vasoconstriction. The systemic and portal venous pressures were elevated in this group and this was thought to be a compensating mechanism to enable normal fluid filtration to take place through a reduced vascular bed.

There was no significant difference in the loss of total protein and T1824 labelled protein in the plasma in any of the groups. The amount of T1824 recovered in the lymph was not significantly different in any of the groups. The thoracic duct protein concentration was shown to be affected by qualitatively different factors in the three groups.

The equilibrium between specific activities of plasma and lymph was attained much more rapidly even with low net infusions in the anoxia and noradrenaline groups than in the air group. Evidence was presented that this was due to a reduction of the effective size of the extravascular fluid and protein pool, and that this phenomenon accompanied systemic vasoconstriction.

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STUDIES IN CARBON TETRACHLORIDE POISONING

I. THE DETECTION AND ESTIMATION OF CREATINE AND RELATED COMPOUNDS

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In carbon tetrachloride poisoning there is an increased excretion of creatine (Hove, 1949). Before a detailed study could be undertaken of changes in creatine levels in the tissues, it was necessary to modify the diacetyl method (Eggleton, Elsden and Gough, 1953) for the estimation of this compound. Compounds containing sulphydryl groups interfere with this reaction and must be removed. Chloromercuribenzoic acid is satisfactory for this purpose when the molar concentration of sulphydryl groups is less than ten times that of the creatine concentration (Ennor and Stocken, 1948). The increased amounts of mercury compounds required to bind higher concentrations of —SH groups interfere with the diacetyl reaction. In the present investigation preliminary removal of the sulphydryl containing compounds was effected by chromatography and this process was the most satisfactory among those tried.

As arginine and glycocyamine react with diacetyl, it is necessary to have an independent method for their estimation. Arginine and glycocyamine form a red coloured complex on the addition of a solution of α-naphthol or 8-hydroxyquinoline followed by alkaline hypobromite (Sakaguchi, 1925, 1950). The specificity of this reaction was re-examined and its sensitivity compared with the diacetyl reaction. Both tests were applied to the detection of arginine and the other compounds on paper chromatograms.

MATERIALS AND METHODS.

Albino rats were kept on the stock breeding diet which contained 22 p.c. of protein. When the animals reached about 300 gm. body weight, they were killed with coal gas and the tissues were used for chemical estimations without delay.

When urine samples were required the rats were placed in metabolism cages. In order to preserve the urine the glass funnels of the cages were sprayed daily with 5 ml. of a solution of benzoic acid (3 p.c.) and phenylmercuricnitrate (0·05 p.c.) in 50 p.c. alcohol.

The following methyl derivatives of guanidines were used:

monomethylguanidine sulphate, *s*—dimethylguanidine iodide, *as*—dimethylguanidine sulphate, *s*—trimethylguanidine sulphate, *as*—trimethylguanidine iodide, *s*—tetramethylguanidine iodide, *as*—tetramethylguanidine iodide.

[1]Working under a full-time grant from the National Health and Medical Research Council. This investigation was also supported by apparatus grants from the Commonwealth Research Committee, University of Sydney.

Liver and kidney.

Creatine estimation.

Reagents required:

$HClO_4$ (10 p.c.), KOH (saturated), Ethanol (75 p.c.), α-naphthol (1 gm. in 100 ml. of a solution containing 6 p.c. NaOH and 16 p.c. Na_2CO_3), Diacetyl (0·05 p.c.).

Procedure. In a glass homogenizer (Humphrey, 1946) 0·5 gm. of liver or kidney was ground with 1 ml. of $HClO_4$ (10 p.c.). The homogenizer and pestle were rinsed with 1 ml. of 5 p.c. acid. The combined homogenate and washings were centrifuged for 5 minutes at 1,500×*g*. The supernatant was decanted and the residue re-extracted with 0·5 ml. of 5 p.c. perchloric acid. The perchloric acid in the pooled extracts was converted to the insoluble potassium salt by adjusting the pH to 8 with saturated KOH using thymol blue as an indicator and cooling the solutions in an ice bath for an hour. The precipitate was removed by centrifuging. Aliquots of the supernatant containing 0·5 to 5 μg. of creatine were transferred to strips of Whatman No. 1 filter paper (30×1·5 cm.). For liver extracts it was necessary to transfer as much as 100 μl.; evaporation of the solvent on the paper was aided by a fan.

The tanks used had a maximum capacity of 48 strips. The developing solvent, 75 p.c. ethanol, was allowed to flow down the paper. Separation of creatine from the interfering compounds was complete in 4 hours, after which the strips were dried in air. The Rf value of creatine was checked on at least two strips by the use of the following procedure: two volumes of freshly prepared α-naphthol and one volume diacetyl solutions were mixed, and a thin film of this mixture was spread out on a glass plate. The dried strips were momentarily dipped, stretched out into this solution and then transferred onto a white tile. It was found that this method of locating spots was more sensitive and gave more uniform results than spraying.

Five cm. pieces of replicate strips centred at the Rf value were cut out and were washed in 1 ml. of water for 15 minutes with occasional shaking. Of the supernatant 0·7 ml. was withdrawn, and to this was added 0·2 ml. of α-naphthol solution followed by 0·1 ml. of diacetyl solution. The red colour which developed was read 30 minutes after the addition of the reagents in a photo-electric colorimeter with an Ilford filter, No. 604, having a maximum transmittence at 520 mμ. Some unknown water-soluble compounds in the paper increased the colour of the blank, hence the standard reference curve was established by taking the standard solutions through the whole procedure.

Arginine and glycocyamine estimation.

Reagents required:

Trichloracetic acid (10 p.c.), Arginase preparation (Höbermann, 1947), α-naphthol (0·04 p.c. in ethanol), Urea (16 p.c.), Hypobromite Solution I (0·4 ml. of bromine in 100 ml. of 5 p.c. NaOH), Hypobromite Solution II—used only for chromatography (0·1 ml. of bromine in 100 ml. of 5 p.c. NaOH).

Procedure. A sample of 0·5 gm. of liver or kidney was homogenised with 1 ml. of water, the homogenizer and pestle rinsed with 1 ml. of water, and to the combined homogenate and washing was added 1 ml. of arginase solution. The extracts were deproteinized with 3 ml. trichloracetic acid (10 p.c.) either at once or after incubation at 37° C. for 45 minutes. The tubes were then placed in the refrigerator for 30 minutes before centrifuging. The residue was re-extracted with 1 ml. of trichloracetic acid (5 p.c.). The pooled extracts were

neutralized to pH 7, using bromthymol blue as an indicator, boiled for 4 minutes to coagulate the remaining proteins, cooled, filtered and diluted to 10 ml.

Solutions containing 2 to 50 μg. arginine or glycocyamine were diluted to 5 ml., and to these were added 1 ml. of urea solution and 1 ml. of α-naphthol solution. The tubes were cooled in an ice bath until the temperature of the solution was 6° to 7° C. Sufficient mixing was obtained by bubbling air through each tube during the addition of 1 ml. of alkaline hypobromite solution. Aeration was continued for a further 10 minutes. The red colour which developed was read 30 minutes after the addition of the hypobromite.

When it was necessary to detect arginine and glycocyamine on paper, urea was not included with the reagents. Urea is normally used to remove excess hypobromite which otherwise destroys the coloured complex of the Sakaguchi reaction. In this process urea and the hypobromite form a yellow compound which, on paper, made it difficult to detect the red Sakaguchi spots. The necessity for urea spraying was obviated by reducing the hypobromite concentration to make the red colour more stable.

Detection of other compounds on chromatograms.

The nitroprusside test of Toennies (1951) or the azide test of Williams (1951) was used for the detection of —SH groups. For the picric acid test of creatine the papers were first dried at 100° C. for 6 hours in order to convert the creatine to creatinine and then dipped into a solution of picric acid (1·2 p.c.) mixed with equal volumes of NaOH (1·7 p.c.) immediately before use.

Serum.

Creatine estimation.

As serum contains no appreciable amounts of compounds which interfere with the diacetyl reaction, there was no need to chromatogram these samples. Protein free filtrates were prepared as for arginine estimation and creatine was determined on an aliquot.

Arginine and glycocyamine estimation.

One ml. of serum diluted to 2 ml. with water and one ml. of arginase solution were added, and then the procedure was the same as for liver.

Urine.

Only creatine and glycocyamine were estimated on urine samples. The 24-hour specimens were diluted to 50 ml. and then centrifuged. Trichloracetic acid was used to remove any traces of protein present.

For chromatography the urine samples were used as collected without any further treatment. Water saturated phenol and a mixture of butanol acetic acid (Williams, 1951) were used as solvents in addition to 75 p.c. ethanol.

EXPERIMENTAL.

Sensitivity of the diacetyl and the Sakaguchi reactions.

The Sakaguchi reaction was found to be more sensitive than the diacetyl reaction (Table 1). Readings were taken 30 minutes after the addition of the last reagent. The rate of colour development varied; with creatine the maximum

colour was obtained at 15 minutes, whereas the other compounds reacted more slowly. With methylguanidine the diacetyl reagent gave a colour which was still increasing at 120 minutes.

If solutions more dilute than 10^{-5} M were used, the various compounds gave proportionately less diacetyl colour than creatine; hence it was considered inadvisable to have a fixed correction factor as recommended by Melville and Hummel (1951), but to establish standard reference curves for each compound and to make the necessary corrections with the aid of these.

Less than 1 μg. of all the compounds listed in Table 1 could be detected on paper chromatograms. The two methods were about equally sensitive on paper but the diacetyl reaction was more convenient as all the reagents could be mixed before dipping the papers. However, the Sakaguchi reaction was especially useful in distinguishing glycocyamine from creatine as their Rf values tended to be similar in many solvents. The above two methods were more sensitive than either the picric acid or the ninhydrin test.

TABLE 1.

Comparison of the sensitivity of the diacetyl and Sakaguchi reactions.

Compound	Molecular weight	Diacetyl reaction (Optical density of 10^{-5} M solutions)	Sakaguchi reaction (Optical density of 10^{-5} M solutions)
Creatine	131	0·107	—
Arginine	174	0·042	0·115
Glycocyamine	117	0·033	0·176
Methylguanidine	73	0·015	0·242

TABLE 2.

Rf values of pure solutions at pH 7.

	75 p.c. ethanol
Creatine	0·50
Arginine	0·17
Glycocyamine	0·40
Methylguanidine	0·77
Glutathione	0·32
Oxidised glutathione	0·11

Specificity of the Sakaguchi reaction.

Sakaguchi (1925) has suggested that only monoguanidines react to give the characteristic red colour. Poller (1926), on the other hand, has claimed that *s*-di- and tri-methylguanidines also form red coloured complexes. In the present experiment all the seven possible mono-, di-, tri- and tetra-methylguanidines were examined for their participation in the Sakaguchi reaction (for the list of compounds see section on Materials). In no case was a red colour obtained

even if the molar concentrations were a hundred times those normally used. Evidence of the purity of these compounds has been reported (Angyal and Warburton, 1951). Unfortunately, no such data were given by Poller. The red colours he obtained might have been caused by traces of methylguanidine in his samples.

Problems of chromatography and tissue extraction.

A solution of 75 p.c. ethanol proved satisfactory for separating glutathione from creatine. It also gave very good separation of arginine from glycocyamine and methylguanidine (see Table 2). The actual Rf values of the various compounds did vary slightly from experiment to experiment, depending on the temperature. Much larger variations were observed with changes of pH and salt concentration of the solutions to be chromatogrammed. The Rf values of most compounds were increased if the extracts were either strongly acid or alkaline or if the salt concentration was high. Under these conditions separations were not obtained. If high concentrations (e.g., 10 μg. per spot of glycocyamine or methylguanidine) were used after running the chromatograms a second spot appeared just behind the first one. Multiple spots of a single compound, presumably due to the presence of more than one ionic form, have been previously reported (Lauden and Awapara, 1951). Thus, when identifying spots it was necessary to control the pH and use solutions containing low concentrations.

The main difficulty in developing a quantitative method for creatine estimation was to obtain an extract of approximately neutral pH and of relatively low salt concentration. As the creatine level in livers is low, it was necessary for each spot to contain the extract of an equivalent of 30 mg. of wet tissue. Trichloracetic acid extraction is generally used when estimating creatine in tissues. When such extracts were chromatogrammed variable Rf values were obtained. The spots were diffuse and the separation of creatine from glutathione was not always achieved. Neutralizing the extracts prior to chromatography did not overcome all these difficulties. Trichloracetic acid extracts have been successfully used for chromatography (Feldman and Gunsalus, 1950), but the amount of acid applied to the paper was only about a tenth of that used in the present work.

Perchloric acid extraction was found to be the most satisfactory method among those tried. More creatine was obtained in one perchloric acid extraction than in three alcohol ones. Very little creatine was found in the second perchloric extraction and none in the third. The excess perchloric acid was removed as its insoluble potassium salt. Each stage was checked for losses and the average recovery was 97 p.c. The standard error was highest for liver estimation; for 12 liver extracts, containing 1·56 mg./100 gm. of tissue, it was ±0·37. In biological experiments, for which this method was developed, changes of 100 to 300 p.c. occurred.

Although perchloric acid extraction was most suitable for creatine estimation, in the case of glycocyamine only 30 to 50 p.c. was extracted when compared with extracts prepared by water homogenization followed by trichloracetic acid precipitation. As creatine is present in the cytoplasm (Cohen, 1953), whereas glycocyamine occurs in the mitochondria (Sakar, Beinert, Fuld and Green, 1952), this may explain why the latter is less readily extracted if extraction and precipitation of protein are carried out at the same time.

Nature of compounds present.

In preliminary experiments rat urines were preserved with benzoic acid. On chromatogramming these urines Sakaguchi spots were observed which could not be identified. It was found that benzoic acid was not sufficient to prevent bacterial growth and phenylmercuricnitrate was included in the urine preservative. In well preserved samples which had not been precipitated with trichloracetic acid, four Sakaguchi positive spots were obtained. Three of these were identified with re-inforcement tests to be arginine, glycocyamine and methylguanidine. The fourth spot had an Rf value of 0 in all solvents tried. After hydrolysis this spot disappeared and the arginine spot became more intense. This suggests that the compound is a protein or a polypeptide containing arginine. A peptide, γ-glutamylarginine, has been found in kidney extracts (Kinoshita and Ball, 1953), but the Rf value in phenol for this dipeptide was 0·67 as compared with 0 for our compound.

Arginine, creatine, glycocyamine and methylguanidine were also detected in the tissue extracts of albino rats. The concentrations of these compounds in urine, serum and tissues are discussed in the following paper.

SUMMARY.

The specificity and sensitivity of the diacetyl and Sakaguchi reactions were re-investigated. These reactions were applied to paper chromatography. The effect of pH and other conditions on the Rf value of creatine, arginine, glycocyamine and methylguanidine were studied.

Glycocyamine and traces of protein, arginine and methylguanidine were identified in the urine of rats. The presence of these compounds together with creatine was also observed in tissue extracts.

A satisfactory method using paper chromatography for separating creatine from interfering compounds was also developed.

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STUDIES IN CARBON TETRACHLORIDE POISONING

II. THE EFFECT ON CREATINE METABOLISM

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Hove (1949) has found that carbon tetrachloride poisoning in rats produces some of the symptoms of vitamin E deficiency. In both these conditions there is an increased excretion of creatine in the urine. However, creatinuria has also been observed in fasting, hypothyroidism and many other conditions (Smith, 1951). Loss of creatine from the muscles, impaired renal function (Smith, 1951) and an increased synthesis of creatine (Melville and Hummel, 1951) have all been suggested as possible causes of creatinuria.

In the present investigation changes in the concentrations of creatine and its precursor glycocyamine were studied in carbon tetrachloride injected rats. Animals fasted for 24 hours were also included for comparison because those injected had a reduced appetite and therefore would be expected to excrete more creatine.

MATERIALS AND METHODS.

Male albino rats were kept on the stock breeding diet which contained 22 p.c. protein. When the animals reached 300 gm. body weight, they were injected intraperitoneally with 0·3 ml. of carbon tetrachloride.

Creatine, glycocyamine and arginine were estimated as previously described (Eden, Harrison and Linnane, 1954). The method of Peters (1942) was used for creatinine.

TABLE 1.

Creatine levels in serum, kidney and liver.

Treatment	No. of animals	Liver weight (gm.)	Liver creatine (mg./100 gm.)	Liver creatine (total μg.)	Serum creatine (mg./100 ml.)	Kidney creatine (mg./100 gm.)
Control	12	9·94±1·04*	2·58±0·04*	257± 58*	4·19±0·85*	16·1 ±4·4
24 hours fasted	12	8·95±0·85	4·73±1·35	421±133	6·29±1·50	49·11±7·68
CCl_4 injected	12	11·43±1·65	4·70±1·54	531±201	5·18±0·05	37·45±9·16

* Standard deviation of the mean.

[1]Working under a full-time grant from the National Health and Medical Research Council. This investigation was also supported by apparatus grants from the Commonwealth Research Committee, University of Sydney.

EXPERIMENTAL.

Preliminary experiments indicated that 12 to 48 hours after injection an increased concentration of creatine was present in the liver and kidneys. As the maximum concentration occurred at 24 hours, this was the time interval chosen for the main experiment. In the urines the maximum was observed at 48 hours, one day later than the tissues.

Both fasting and carbon tetrachloride poisoning significantly ($P<0{\cdot}001$) increased the concentration of creatine in the liver (see Table 1 and Fig. 1). Increased creatine levels in the livers of carbon tetrachloride poisoned animals have been previously reported (Ennor and Stocken, 1948; Hove and Hardin, 1952). In some of their experiments the animals were fasted prior to killing so the increase might have been caused by either or both factors. There was an increase in the total liver weight of carbon tetrachloride injected animals but a decrease with fasted animals; it seems, therefore, more suitable to compare results expressed as total creatine per liver. On this basis the livers of the carbon tetrachloride groups contained 26 p.c. more than the fasted ones, but this difference is still not statistically significant ($P>0{\cdot}05$).

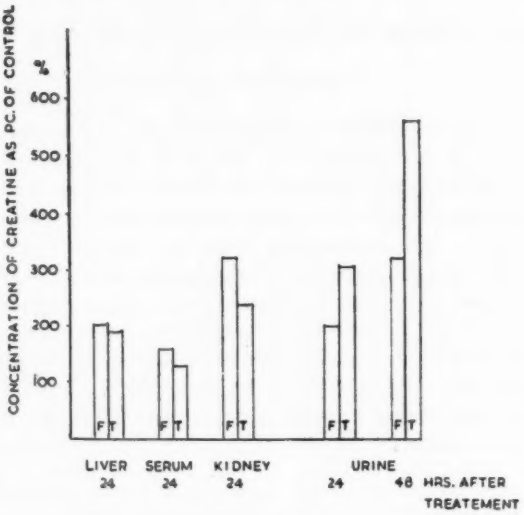

FIG. 1. CHANGES IN CREATINE CONCENTRATION FOLLOWING CCl_4 INJECTION OR FASTING

F 24 HRS. FASTED ANIMAL.
T CCl_4 INJECTED ANIMAL

The increases in serum and kidney creatine were more marked in the fasted group ($0{\cdot}01>P>0{\cdot}001$). In the urines this relationship was reversed and the carbon tetrachloride animals execreted more creatine than the fasted animals ($0{\cdot}02>P>0{\cdot}01$).

Arginine and glycocyamine are precursors in the synthesis of creatine and changes in their concentrations were also studied. Although the glycocyamine concentrations in the livers of carbon tetrachloride injected animals were found to be lower than those of the control or fasted animals, when expressed as total

per liver this difference disappeared (see Table 2). Similarly, no changes were observed in the serum levels. A slight drop was noted in the glycocyamine excretion 24 hours after treatment, but this change was not statistically significant ($P>0{\cdot}05$, Table 2).

Liver and urine arginine levels were too low to be estimated by the present method. In the case of kidney, variable amounts of arginine were obtained, but never more than 3 mg./100 gm. Using *Strep. faecalis* for arginine determination Solomon, Johnson, Sheffner and Bergein (1951) found 24 mg. of arginine/100 gm. of rat kidney. No figures were given for the concentration of glycocyamine and the authors do not state whether this organism was able to distinguish between arginine and glycocyamine. In the present experiments no changes were observed in the serum arginine levels (Table 2).

TABLE 2.

Glycocyamine and arginine levels.

Treatment	No. of animals	Liver glycocyamine (mg./100 gm.)	Liver glycocyamine (total μg.)	Serum glycocyamine (mg./100 ml.)	Kidney glycocyamine (mg./100 gm.)	Serum arginine (mg./100 ml.)
Control	6	3·5±0·3*	350±50*	0·84±0·23*	19·7±3·2*	1·84±0·90*
24 hours fasted	6	3·4±0·35	310±32	0·80±0·26	20·9±5·3	1·94±0·34
CCl_4 injected	6	2·8±0·57	310±62	0·76±0·16	17·7±2·2	1·86±1·26

* Standard deviation of the mean.

Every 24 hours about 2 p.c. of the total body creatine is irreversibly changed to creatinine (Bloch, Schoenheimer and Rittenberg, 1941) and excreted in the urine. If a smaller amount of creatine is converted to creatinine a decreased excretion of the latter compound might be expected. Such a decrease following carbon tetrachloride injection has been reported (Hove, 1949). A slight decrease was also observed in the present investigation (see Table 3).

TABLE 3.

Creatine, glycocyamine and creatinine excretion.

Day	No. of animals	Creatine (mg./day)	Glycocyamine (mg./day)	Creatinine (mg./day)
0 F	10	2·6±1·4*	3·2±0·8*	12·3±2·5*
0 T	10	1·4±1·0	2·5±0·8	14·9±1·0
1 F	10	4·0±1·9	1·8±0·4	11·6±1·0
1 T	10	6·1±5·4	2·0±0·9	13·3±2·6
2 F	10	7·4±2·6	3·2±0·9	11·3±2·8
2 T	10	11·1±6·3	2·1±0·7	11·8±1·7
8 F	10	2·2±1·6	3·2±0·5	14·0±2·6
8 T	6	2·5±1·7	3·6±0·3	11·3±2·4

F=fasted. T=carbon tetrachloride injected animals. * Standard deviation of mean.

DISCUSSION.

Melville and Hummel (1951) suggest that an increased synthesis is the cause of the increased concentration of creatine observed in the liver of vitamin E deficient animals. In carbon tetrachloride poisoning there is also an increased concentration of creatine in the liver, but liver slices from these animals actually had a decreased ability for converting added glycocyamine and methionine to creatine (Hove and Hardin, 1952; Richter, 1951). High liver levels do not necessarily prove an increased synthesis. Calculations based on the data reported by Heinrich and Matill (1949) show that the total amount of liver creatine is re-synthesized about every 3 hours. Thus an increase of 100 p.c., as observed in the present experiment (Fig. 1), could be caused by a slower rate of removal of the newly formed creatine by the muscles. An impaired ability of these tissues to do so has been shown to occur in muscular dystrophy, another condition in which creatinuria is present (Roche, Benedict, Yu, Bien and Stetten, 1952). To prove their point Roche *et alii* used isotopic creatine. Without the use of labelled creatine it is difficult to decide the exact cause of the increased concentration of creatine in the livers of carbon tetrachloride treated animals. The available facts suggest, however, that an impaired ultilization of the newly synthesized creatine is more likely than an increased synthesis.

Normally, nearly all the creatine in the glomerular filtrate is reabsorbed by the cells on the kidney tubules. Creatinuria would be expected to occur if these cells are damaged. Such damage is present in carbon tetrachloride poisoning (Smith, 1951). No details are given by Smith on the effect of 24 hours fasting on kidney function. The higher kidney and the lower urine creatine levels observed in the present experiments indicate that in fasting the reabsorption mechanism is more efficient than in carbon tetrachloride poisoning.

The initial cause of altered creatine metabolism may well be the same in fasting and in carbon tetrachloride poisoning. The kidney damage of the latter on the other hand may explain the differences observed with these two treatments.

SUMMARY.

When adult rats were injected with carbon tetrachloride or fasted for 24 hours, the concentration of creatine in the liver was nearly doubled. Creatine levels were also increased in the serum, kidney and urine. The serum and kidney levels were higher in fasted animals, whereas the urine levels were higher in animals injected with carbon tetrachloride. No statistically significant changes were observed in the glycocyamine levels of liver, serum, kidney or urine.

Impaired renal function and decreased utilization by the tissues of the newly synthesized creatine are discussed as the possible causes of the changes observed.

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FACTORS AFFECTING THE LYSIS OF ERYTHROCYTES TREATED WITH STAPHYLOCOCCAL β TOXIN

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(Accepted for publication 3rd December, 1953.)

The action of staphylococcal β toxin on the erythrocytes of a number of species of animal was described by Bryce and Rountree (1936). These workers found that when the β toxin was mixed with the erythrocytes of certain species and the mixture held at 37° C. for one hour there was no lysis, but if it was then transferred to the cold the cells lysed. Christie, Atkins and Munch-Petersen (1944) showed that this lysis could be completed in ruminant erythrocytes at 37° C. by the action of a factor produced during the growth of *Streptococcus agalactiae*. Wilson and Slavin (1950) and Murphy, Stuart and Reed (1952) showed that certain other organisms, notably some strains of *Streptococcus uberis*, gave the same reaction, whilst not all strains of *Strep. agalactiae* did so. Murphy *et al.* designated the factor responsible for this reaction CAMP. This factor has no demonstrable effect on cells not treated with β toxin.

During the development in this laboratory of a "spot" plate test to detect *Strep. agalactiae* in contaminated cultures, and a tube test for the assay of CAMP, several interesting reactions were observed. These reactions form the basis of the work described in this paper.

Materials and Methods.

Staphylococcal β toxin.

This was prepared from a growth of the pure β toxin producing staphylococcus S32A of Bryce and Rountree, kindly supplied by E. Munch-Petersen. A sloppy agar culture of S32A, incubated for 48 hours at 37° C. in an atmosphere of 20 p.c. CO_2 was filtered through muslin, centrifuged at about 3,500 r.p.m. for 20 minutes, and filtered through a Seitz E.K. pad. The filtrate, referred to as β toxin, was stored in bottles at 4° C.

Standardization of β toxin.

Serial double dilutions of β toxin were prepared in 0·5 ml. quantities in normal (physiological) saline. To each tube was added 0·5 ml. of a suspension of 3 p.c. washed sheep erythrocytes in a 1 in 4 dilution in saline of neutral CAMP factor. Lysis in this system was found to run parallel with titration of β toxin by the usual "hot-cold" method. The last tube showing complete lysis was taken as the end point.

CAMP factor.

This was produced by growing *Strep. agalactiae* in crystal violet broth. For stock CAMP factor our strain 519/13/1 was used, the culture being filtered through a Seitz E.K. pad and the filtrate stored in the cold.

Crystal violet broth.

The medium used for the isolation and growth of *Strep. agalactiae* was 2 p.c. peptone (B.D.H.), 1 p.c. Lemco, 0·1 p.c. glucose and 0·5 p.c. sodium chloride with crystal violet to a final concentration of $1:1{\cdot}6\times10^6$ and at pH 6·8. This was autoclaved at 10 lb. pressure for 15 minutes.

EXPERIMENTAL.

Effect of staphylococcal β toxin on horse erythrocytes.

Horse blood is the standard blood in use in this Institute for routine media and was not mentioned in Bryce and Rountree's work either as reacting or not reacting to β toxin. Because of the wide use of this reagent in media, it was tested by growing the staphylococcus S32A on horse blood agar plates and in tube tests with β toxin for hot-cold lysis and for CAMP lysis. No lysis was produced in any of these experiments.

Effect of saline concentration on lysis of β toxin sensitized sheep cells.

When a drop of culture of *Strep. agalactiae*, or a Seitz filtrate of such a culture, is placed on the surface of a sheep blood agar plate containing β toxin, lysis occurs in a few minutes, giving a clear spot in an otherwise unchanged plate. It was found that certain batches of crystal violet broth gave this reaction before inoculation, and it was considered that the effect might have been due to variations in the osmotic pressure of the media.

A range of saline dilutions and sensitized blood agar plates, to represent media of varying osmotic pressures, was prepared as follows: Quantities of 8N saline, 6·6 ml., 6·0 ml., 5·5 ml., 5·0 ml., 4·5 ml., 4·0 ml., 3·6 ml., 3·2 ml. and 2·8 ml. were made up to 10 ml. with distilled water. Each dilution was added in 5 ml. amounts to 15 ml. distilled water, and also to 13·5 ml. melted 2 p.c. agar in distilled water. To each agar-saline dilution was added 0·5 ml. β toxin and 1 ml. sheep blood, and the whole, after mixing, was poured into petri plates. The blood agar plates together with the saline dilutions were held for 20 minutes at 37° C. Using a nichrome loop, one drop of each saline dilution was then placed on the surface of each plate. The plates were replaced in the incubator for one hour and examined for lysis.

The results, together with the final salt concentration of the plates and the saline dilutions, are given in Table 1. The toxin and blood, being isotonic with normal saline, contribute a slight additional osmotic effect to the agar plate equivalent to 0·06 p.c. saline. Some lysis, uniform throughout the plate, occurred with the lower salt concentrations, but even in the plates of the lowest salt content sufficient cells survived for the reaction to be clearly observed. It is seen from the Table that where the saline spot was isotonic with the agar there was no lysis. Where the saline was slightly hypotonic, or markedly hypertonic, lysis occurred.

TABLE 1.

The effect of applying hypotonic and hypertonic saline to the surface of toxin sensitized sheep blood agar plates of varying salt concentration.

Percentage salt concentration in plates	Percentage salt concentration of applied liquid.													
	6·80	1·70	1·53	1·36	1·22	1·12	1·02	·93	·85	·77	·68	·61	·54	·48
1·18	—	—	—	—	—	—	1	2	2	2	2	2	2	2
1·08	1	—	—	—	—	—	—	1	2	2	3	3	3	3
·99	2	—	—	—	—	—	—	—	1	2	3	3	3	3
·91	2	1	—	—	—	—	—	—	—	1	2	3	3	3
·83	2	2	1	—	—	—	—	—	—	—	1	3	3	3
·74	2	2	1	1	—	—	—	—	—	—	1	2	3	3
·67	3	3	3	2	2	1	—	—	—	—	—	1	2	3
·60	3	3	3	3	3	2	2	1	—	—	—	—	1	2
·54	3	3	3	3	3	3	3	3	1	—	—	—	—	1

—=no lysis. 1=lysis just barely perceptible. 2=lysis marked. 3=lysis complete in area of spot.

These results were typical of a number of experiments where sensitized blood agar plates were prepared, using crystal violet broth of various salt concentrations and applying saline dilutions or broths of various salt content. No lysis occurred on plates prepared without β toxin except with very dilute saline.

All attempts to reproduce the lytic effect of hypertonic saline in β toxin-sensitized cells in liquid media were unsuccessful, although sensitized cells were found to be lysed more readily by hypotonic saline than were normal cells.

Effect of pH on sensitization of sheep erythrocytes and on subsequent lysis.

An accidental observation that sheep red cell-β toxin mixtures were sometimes lysed by changes in pH made it necessary to examine more closely the course of the sensitization reaction and subsequent behaviour to changes in the pH of the suspending medium.

It was found that fully sensitized cells at pH 7·3 could be rapidly and completely lysed by the rapid addition of sufficient hydrochloric acid to reduce the pH to 7·05. Double the quantity of acid added over 12 minutes reduced the pH to 6·5, but failed to lyse the cells. Provided the acid and cells were mixed quickly, the initial pH was found to have little effect on subsequent lysis except where conditions were unsuitable for the action of the β toxin. Normally, with the addition of 0·05 ml. of 0·01 N HCl to 0·5 ml. of fully sensitized cells lysis was complete in a few seconds. The sensitized cells were found to be very much less susceptible to a rising pH, but a large sudden rise from pH 6·8 to 7·6 in a few seconds caused almost complete lysis. Because of the very rapid lysis on the addition of acid, this technique was found to be particularly suited to a study of the course of sensitization of the cells by β toxin. Furthermore, any sensitization taking place after the addition of the acid did not produce further lysis.

The effect of the interaction of β toxin concentration, pH and temperature on the rate of sensitization of sheep red cells was demonstrated in the following experiment.

Staphylococcal β toxin, titre 1:256, in 1 ml., 0·5 ml., 0·25 ml. and 0·08 ml. quantities were added to four tubes containing 10 ml. of 0·2 p.c. phosphate buffer, 2 of which were at pH 7·7 and 2 at pH 6·0. One tube of each pair was heated to 37° C. in the incubator and the other left at room temperature, 17° C. A suspension of 6 p.c. washed sheep red cells was adjusted to each of these two temperatures. At zero time 10 ml. of isothermic cell suspension were added to each tube of β toxin-buffer mixture. At 2½, 10, 30, 60 and 120 minutes 3 ml. were withdrawn from each tube and 0·5 ml. put into each of 6 small test tubes previously prepared containing 0 to 5 drops of N/100 HCl made up to 5 drops with saline and adjusted to the temperature of the test. Two minutes after mixing the tube with least acid showing complete lysis was recorded.

TABLE 2.

Effect of pH, temperature and concentration of toxin on the rate of sensitization of sheep red cells.

pH of cell β toxin mixture	M.H.D. β toxin	Temperature of sensitization: 37° C. Time sensitized in minutes					Room temp. 17° C. Time sensitized in minutes				
		2½	10	30	60	120	2½	10	30	60	120
7·4	12	3	4	4	4	4	—	—	3	4	4
	6	—	3	4	4	4	—	—	2	3	3
	3	—	—	3	4	4	—	—	—	1	1
	1	—	—	—	—	3	—	—	—	—	—
6·3	12	—	—	2	4	4	—	—	—	1	1
	6	—	—	—	3	4	—	—	—	—	—
	3	—	—	—	—	3	—	—	—	—	—
	1	—	—	—	—	—	—	—	—	—	—

The figures represent the number of the tube containing the least acid in which haemolysis is complete. Tube 1 contains 5 drops of N/100 HCl in N saline; tube 2, 4 drops; tube 3, 3 drops; tube 4, 2 drops; tube 5, 1 drop; and tube 6, the control, no acid. All tubes were made up to 5 drops with N saline. 1 drop is 0·027 ml.

The results are set out in Table 2. It will be seen that the pH, concentration of β toxin and temperature all affect the rate of sensitization of the cells. At 37° C. and pH 7·4 sensitization was very rapid, and with 12 M.H.D. of β toxin was almost complete in 2½ minutes. A reduction of the temperature to 17° C., the pH to 6·3 or the β toxin concentration to 3 M.H.D. produced a marked slowing of the reaction, sensitization requiring 30 minutes or more. If two or more of these factors were reduced together the sensitization time was very greatly extended. If the pH was reduced to 6·3 and the temperature to 17° C. practically no sensitization took place in 2 hours, even if the amount of β toxin remained constant at 12 M.H.D. A similar result was obtained if the pH was kept at 7·4 but the temperature and quantity of β toxin was reduced.

DISCUSSION.

These observations show that, although erythrocytes sensitized to β toxin are stable if their environment remains unaltered, they are in fact exceedingly fragile. In addition to the hot-cold and CAMP factor lysis described by previous workers, these cells may be lysed by alteration of either the pH or the osmotic pressure of the suspending medium. It has been shown that the cells, although more sensitive to a falling than to a rising pH, may be lysed by either, and that the lysis is dependent on rate of change of pH rather than the magnitude of the change. The sensitized cells are more sensitive to a lowering of the salt content of the suspending medium than normal sheep cells. The reason for the lysis in an agar medium when hypertonic saline is applied to the surface is not clear. The lysis induced by hypotonic and hypertonic saline relates to the osmotic pressure of the medium at the time of sensitization and not to physiological normality. For example, some of the cells sensitized in agar in 1·18 p.c. saline are lysed on the application of 1·02 p.c. saline, while those surviving immersion in 0·42 p.c. saline and sensitized in this medium are lysed on the application of 0·61 p.c. saline.

Where streptococcal cultures or filtrates are applied to sensitized red cells in agar plates, the agar should be slightly hypotonic with respect to the applied liquid. This is achieved in practice by preparing broth and agar from the same batch of medium and adding 10 p.c. of distilled water to the agar.

The common use of a slightly alkaline reaction in media for the growth of staphylococci usually produces β toxin at a pH suitable for rapid action with the erythrocytes. The present work shows that this action may be very considerably retarded by reduction of the pH or of the temperature of the suspending medium.

The effects recorded in this work are of importance where the production of CAMP factor is being studied quantitatively or where the CAMP staphylococcal β toxin reaction is induced by the application of the streptococcal factor to previously sensitized cells. Cells must be fully sensitized if observations of CAMP lysis are to be quantitative. If, however, sensitization is complete before the addition of the streptococcal growth lysis may occur from salinity or pH effects.

In tube haemolytic titrations of CAMP factor, if a 3 volume technique is used adding cells last to a mixture of CAMP dilutions and β toxin, the strongly acid cultures of streptococcus will inhibit sensitization and no reaction may be obtained, or if the acid dilutes out more quickly than the CAMP factor a prozone phenomenon would be expected and is in fact found. If the cells and β toxin are mixed and added to CAMP dilutions the same effect is observed where the pH of the mixture is low or the room temperature is low. In summer and with the cell-β toxin mixture at about pH 7·0 to 7·4 sensitization will be so rapid that, if the cells and toxin are mixed immediately before use, some lysis will be

produced on addition to the more strongly acid cultures whether CAMP factor is present or not. In practice it has been found that reliable titrations are obtained where the cells and β toxin are mixed at pH 7·0 to 7·4 and the streptococcal cuture neutralized before dilutions are prepared.

SUMMARY.

The lysis of sheep erythrocytes sensitized to staphylococcal β toxin by changes in pH and salt concentration of the suspending medium is described.

The interaction of temperature, pH and concentration of β toxin on the rate of sensitization is demonstrated.

The effect of these findings on the observation of the staphylococcal β toxin-streptococcal CAMP reaction is discussed.

Horse erythrocytes are placed in the group of cells not attacked by staphylococcal β toxin.

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SOME FACTORS INFLUENCING THE PRODUCTION OF CAMP FACTOR BY *STREPTOCOCCUS AGALACTIAE*

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The lytic action of *Streptococcus agalactiae* on ruminant erythrocytes sensitized by staphylococcal β toxin was described by Christie, Atkins and Munch-Petersen (1944). This reaction in various modified forms has been used by Wilson and Slavin (1950) and Murphy, Stuart and Reed (1952) in the preliminary screening of organisms isolated from contaminated milk cultures for the presence of *Strep. agalactiae*. These workers have found that not all strains of group B streptococci gave this reaction, and also that certain other organisms, notably some strains of *Strep. uberis*, reacted.

During the development of a screening test for Group B streptococci in this laboratory, it was observed that certain strains produced the factor (CAMP) in abundance, others less freely, and some not at all. The type of medium was also found to have a marked influence on the quantity of factor produced. The work described in this paper arose out of an attempt to determine whether a medium could be evolved in which all strains of *Strep. agalactiae* would produce a detectable amount of the factor.

Smith (1937) showed that the amount of haemolysin produced by Group A streptococci was influenced greatly by the concentration of glucose and of peptone. Okamoto (1937) and Bernheimer and Rodbart (1948) showed that the production of streptolysin "S" by group A haemolytic streptococci was greatly increased by the addition of nucleic acids from various sources to the medium.

Materials and Methods.

Strains of Streptococcus agalactiae.

The strains of *Strep. agalactiae* used in this work were isolated from milk samples submitted for the diagnosis of bovine mastitis, and all gave a positive precipitin reaction with Lancefield's group B serum. They were selected from amongst over 1,000 strains isolated and were mainly those which produced little CAMP factor.

Titration of CAMP factor.

Staphylococcal β toxin was prepared and standardized as described in a previous paper (Pulsford, 1954), in which the pitfalls associated with this titration were discussed. The erythrocyte suspension was prepared by washing sheep red blood cells 3 times in normal saline. The culture of streptococcus was neutralized by the addition of N/5 or N/20 NaOH. to the first blue colour with brom thymol blue. Serial double dilutions of neutral culture were

prepared with 0·5 ml. quantities of saline in 3×⅝ inch test tubes. To each tube was added 0·5 ml. of a mixture of 12 M.H.D. staphylococcal β toxin and 3 p.c. washed sheep red cells freshly mixed from 2 to 4 minutes before addition to the test. The mixture was incubated for 1 hour at 37° C. and then the results recorded.

For the sake of brevity and ease of calculations, results in all haemolytic titrations were expressed as the number of the last tube showing complete lysis. Using serial double dilutions, this figure is related to the concentration of lytic factor in the undiluted medium by the equation $x=\log y/\log 2$ where x is the tube number and y is the concentration in the undiluted medium. This method of expressing the results, in addition to simplifying statistical calculations greatly and reducing the bulk of tabulated data, permits the use of the arithmetic mean to express the average response in a geometric series.

EXPERIMENTAL.

The effect of growth inhibitors on the production of CAMP factor by Streptococcus agalactiae.

Sodium azide. Earlier experiments indicated that sodium azide, crystal violet and glucose, present together in a medium, inhibited the production of CAMP factor. It was thought that the low final pH produced by the growth of *Strep. agalactiae* in the presence of glucose may have been responsible for this effect.

Two batches of Lemco broth containing $1:1{\cdot}6\times10^6$ crystal violet were prepared, one with 0·0025 p.c. sodium azide added and the other without azide. Each was divided into three and the pH adjusted to 7·3, 6·2 and 5·2 respectively. Ten tubes of each medium were inoculated with *Strep. agalactiae* strain 519/13/1, incubated for 24 hours and titrated for CAMP factor. The results are shown in Table 1.

TABLE 1.

The effect of sodium azide on the production of CAMP factor.

pH of medium	Haemolytic titre of 10 cultures in each medium. With sodium azide	Mean	Without sodium azide	Mean
7·3	4 4 4 3 4 5 3 5 4 4	3·8	4 4 4 4 4 4 4 5 4 5	4·2
6·2	4 5 4 4 5 3 5 5 4 4	4·3	5 4 5 5 5 5 5 5 5 5	4·9
5·2	3 3 3 3 3 4 3 3 3 1	2·9	4 4 4 5 5 4 5 4 5 5	4·5

1 p.c. fiducial limit of the means 1·01.

Sodium azide at the concentration used had no effect at the higher pH levels, but less than half the amount of the factor was produced in the medium containing sodium azide at pH 5·2 than in the azide free medium.

Crystal violet. Crystal violet at any concentration less than that which actually inhibited growth, had no effect on the production of CAMP factor. In concentrations of crystal violet which inhibited growth from small inocula, there was no inhibition of CAMP factor production if growth was induced with larger inocula.

The effect of alteration of the basis of the medium.

Preliminary experiments indicated that beef heart infusion broth sown with *Strep. agalactiae* produced considerably less CAMP factor than the same broth which had been concentrated four fold by distillation at a reduced pressure. To determine whether or not this was associated with the accessory substance provided by the meat extract, several media were compared.

Beef heart extract, the same extract concentrated 1:4 by distillation at reduced pressure, 1 p.c. Lab. Lemco and 2 p.c. Lab. Lemco were used as the basis of broth media which were completed by the addition of 1 p.c. peptone, 0·5 p.c. sodium chloride and crystal violet at a final concentration of $1:1{\cdot}6\times10^6$. The pH was adjusted to 7·4 and the medium autoclaved at 15 lb. for 15 minutes. Further beef heart infusion broth was similarly prepared with added acid hydrolysate of casein at concentrations ranging from 1 p.c. to 32 p.c. No differences could be detected in the quantity of CAMP factor produced by streptococcal growth in any of these media.

Two peptic digest media, V.F. and B.V.F.O.S. (Campbell, 1936) and a tryptic digest Hartley's broth (Mackie and McCartney, 1949) were prepared as above, but without added peptone. Production of CAMP factor in these media was irregular, but in no case was there any improvement on the infusion broth.

The effect of peptone concentration.

Increasing peptone concentration of the broth medium increases the quantity of CAMP factor produced as is well seen in the following experiment which is typical of a number of such comparisons.

Two batches of crystal violet beef heart infusion broth were prepared, containing 1 p.c. and 8 p.c. peptone. These were each divided into 5 parts to which nil, 0·1 p.c., 0·2 p.c., 0·3 p.c., and 0·5 p.c. glucose, respectively, were added. The pH was adjusted to 7·4. Five tubes of each medium were inoculated with Strain 519/13/1 and incubated for 24 hours at 37° C. They were then each titrated in serial two-fold dilutions with sheep red cells sensitized to β toxin. The results are shown in Table 2. The effect of glucose will be described more fully below. The maximal production in 8 p.c. peptone occurring with 0·2 p.c. glucose was approximately four times that produced by any glucose level in the 1 p.c. peptone broth.

This effect of peptone was constantly observed except with certain batches of peptone which in the higher concentrations, but sometimes as low as 2 p.c., inhibited growth of the culture. B.D.H. peptone (specially prepared for bacteriological use) was used extensively and was not found to be inhibitory up to 8 p.c. With batches of peptone which were inhibitory, concentration of CAMP factor increased with increasing peptone concentration up to the level where growth was inhibited. This is seen in the following experiment.

TABLE 2.

Influence of peptone level of culture on the concentration of CAMP factor.

Glucose concentration p.c.	Haemolytic titre of 5 cultures in each medium			
	Peptone 1 p.c.	Mean	Peptone 8 p.c.	Mean
nil	5 6 6 6 7	6·0	8 8 8 7 8	7·8
·1	6 6 6 5 5	5·8	8 8 8 7 8	7·8
·2	5 6 5 5 5	5·2	8 8 8 8 8	8·0
·3	5 5 5 5 5	5·0	8 8 8 7 8	7·8
·5	5 5 5 5 4	4·8	7 7 7 7 7	7·0

1 p.c. fiducial limit of means ·66.

Beef heart infusion broth was prepared in 4 batches with 2 p.c. peptone A, 2 p.c. peptone B, 8 p.c. peptone A and 8 p.c. peptone B respectively. Six tubes of each medium were inoculated with strain 519/13/1, incubated 24 hours at 37° C. and titrated. The results are given in Table 3, which shows that the production of CAMP factor from both the peptones was the same at the 2 p.c. level. All the cultures in 8 p.c. peptone B grew well and produced the factor, but only one culture of the 8 p.c. peptone A grew well. This, however, produced a satisfactory level of CAMP factor, while the remainder, which showed varying lesser levels of growth, produced irregular lysis.

TABLE 3.

Inhibitory effect of high levels of certain peptones on the growth and production of CAMP factor.

concentration Peptone p.c.	Haemolytic titres of 6 cultures in each medium			
	Peptone A	Mean	Peptone B	Mean
2	7 6 6 7 7 7	6·6	6 6 6 7 7 7	6·5
8	0*0*4*1*1*8	2·3	7 7 7 8 8 7	7·3

The cultures marked * showed very little growth.
5 p.c. fiducial limit of means 1·9.

The interaction of glucose and pH.

It was noticed in the above experiment that glucose containing media had a marked influence on the production of CAMP factor. Early experiments to demonstrate this effect produced very irregular results, and it was thought probable that the effect was one of acid production preventing the maintenance of an optimal pH for the enzymes producing the factor.

To investigate this, a batch of Lemco broth was prepared containing 2 p.c. peptone, and divided into 6 portions. To three of these 1 p.c. phosphate buffer was added and the pH adjusted to 7·5, 6·8 and 6·0 respectively. The other three were adjusted to the same pH levels, but were not buffered. Each of these

6 media was then divided into 4 portions to which glucose was added to give final glucose concentrations of nil, 0·1 p.c., 0·2 p.c. and 0·5 p.c. respectively. This gave 24 media with pH ranging from 6·0 to 7·4 glucose from nil to 0·5 p.c., and with and without buffer. For each experiment 3 tubes of each medium were inoculated with a strain of *Strep. agalactiae*, incubated 24 hours at 37° C., and the whole culture titrated for CAMP factor and the final pH recorded. Control titrations on uninoculated samples were done from each batch of medium. The experiment was repeated with several strains of *Strep. agalactiae* and again on 1 p.c. peptone broth.

TABLE 4.

Showing the interaction of glucose concentration, original pH and buffer on the production of CAMP factor.

	Mean titre of three cultures							
	With phosphate buffer				Without phosphate buffer			
pH of medium	Glucose concentration p.c.				Glucose concentration p.c.			
	0	·1	·2	·5	0	·1	·2	·5
7·4	·6	1·3	2·0	3·0	1·0	2·0	2·3	1·7
6·8	·6	2·0	3·0	2·7	1·7	3·0	2·3	2·0
6·0	1·0	2·7	2·3	1·7	1·3	2·0	1·7	1·7

1 p.c. fiducial limit of means 1·07.
5 p.c. fiducial limit of means 0·80.

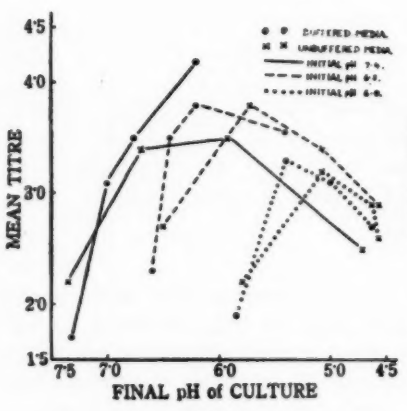

Fig. 1.

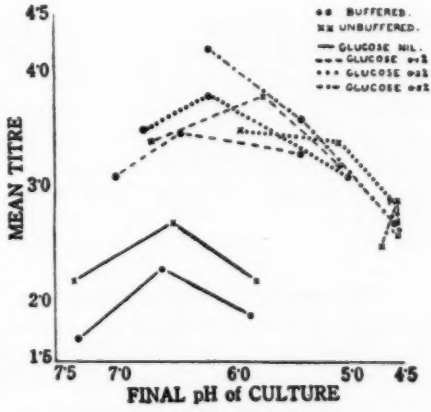

Fig. 2.

Fig. 1. The effect of glucose concentration on CAMP activity and final pH of cultures in various media. Each curve represents medium of one initial pH with or without buffer, the four observations, from left to right, in each curve being 0·0, 0·1, 0·2 and 0·5 p.c. glucose respectively.

Fig. 2. The same data as in Fig. 1, but with each curve representing one glucose and buffer level, the three observations, from left to right, in each curve being the final titre obtained in media with an original pH of 7·4, 6·8 and 6·0 respectively.

The results of one typical experiment are shown in Table 4. It is seen that the optimal glucose level fell as the initial pH fell, but that with the buffer the optimal glucose level was higher in each case than in the unbuffered media. This trend was apparent with all strains and in both 1 p.c. and 2 p.c. peptone broths. The various strains of *Strep. agalactiae* varied in the relative amount of CAMP factor produced, but it has not been possible to demonstrate any essential difference in the pattern of response to glucose, buffer, initial pH and peptone content of the medium. This basic pattern is seen in Fig. 1, in which the mean CAMP titre from 6 experiments, with 3 strains of *Strep. agalactiae* at 2 peptone levels is plotted against the mean final pH of the respective cultures. The four observations in each curve represent the reading at different glucose contents on each medium of the same buffer content and initial pH with the content increasing from left to right. Fig. 2 is drawn from the same data but with the media of the same buffer content and glucose level indicated, each curve representing, from left to right, decreasing initial pH levels. It is seen that optimum production of CAMP factor occurred when the final pH was in the region of 6·0. Above this optimal level the production of factor was directly related to the glucose concentration, while below this level increasing glucose concentration after an initial stimulation serves only to depress further the final pH and to decrease the amount of factor produced.

The effect of nucleic acids.

In view of the spectacular increase in streptolysin S production by group A streptococci in the presence of nucleic acids, it was considered desirable to determine whether a similar increase in CAMP factor occurred. Yeast sodium nucleate (B.D.H.) and material extracted from *Strep. agalactiae* and precipitated in the nucleic acid fraction by the method of Sevag, Lackman and Smolens (1938) were incorporated in media for the production of CAMP factor. No marked increase in CAMP factor production could be demonstrated.

DISCUSSION.

The primary aim of this work was to evolve a medium in which all strains of *Strep. agalactiae* would produce detectable quantities of CAMP factor. While this object has not been achieved, considerable progress has been made in the understanding of the factors controlling the quantity of CAMP produced under various conditions. The more satisfactory media, as will be reported elsewhere, produced detectable amounts of CAMP with a number of strains which were previously regarded as negative, and stronger reactions were obtained with intermediate strains.

The most striking fact that appears from a study of these findings is the number of factors which interact in their effect on the quantity of CAMP factor produced from various culture media.

The effect of crystal violet was negligible up to a concentration which just failed to inhibit growth. Sodium azide was inhibitory, but only when present in media of low final pH. The accessory growth substances provided by meat extracts in the basal medium were shown not to affect CAMP production, either by stimulation or inhibition, nor were they found to be a limiting factor in the usual concentrations employed in media. The reason for irregular results being obtained in digest media has not been investigated because of the difficulties associated with the preparation of a standard product.

The relation of peptone concentration to the amount of CAMP factor produced strongly suggests that the peptone provides material from which the factor is produced. The effect of peptone both with regard to quantity and type is similar to that shown by previous workers with streptolysins of group A streptococcus. Experiments with nucleic acids, however, have demonstrated that the active agent in the peptone is not nucleic acid as it is in the case for group A streptococci (Okamoto, 1937; and Bernheimer and Rodbart, 1948).

The most marked changes in CAMP production were induced by variations in the pH and in the glucose concentrations of the media. A study of Fig. 1 suggests that there are two limiting factors, firstly, the amount of growth in the culture as governed by the quantity of glucose present and, secondly, the adverse effect of low pH. Each curve rises with increasing glucose concentration until the maximum production for that particular pH level is reached, and then with further increase in glucose there is a falling off in CAMP production. A study of Fig. 2 suggests that the optimal pH is in the region of 6·5. This simple interrelation of growth and pH, however, does not satisfy all the observed facts. Firstly, although the presence of buffer held the pH at a higher, and therefore usually a more favourable pH, where there was no glucose and therefore no fall in pH, the buffer had an actual inhibiting effect. Secondly, the unbuffered medium with initial pH of 7·4 almost invariably produced less factor than would be expected, especially at the higher glucose levels. The interpretation of these observations is further complicated by the fact that experiments with hourly samplings of cultures of initial pH 7·4, without buffer and with 0·5 p.c. glucose, show that the cultures start to produce CAMP factor normally, but cease production in the region of pH 6 at about the seventh hour while the culture is still growing vigorously and while other cultures of similar age or pH are producing appreciable quantities of the factor. No hypothesis can be advanced at the present stage to account for these findings.

SUMMARY.

A number of factors in culture media have been investigated for their effect on the production of CAMP factor by *Strep. agalactiae*.

Sodium azide at low pH levels depressed the quantity of factor produced. Increasing peptone levels stimulated production up to the point where growth was inhibited by certain brands of peptone, or up to 8 p.c. where no inhibition occurs.

Accessory growth factors, normally provided in the medium base, and crystal violet have been shown to be without effect.

A complex interaction between the initial pH, glucose content, the buffer level of the medium and final pH of the culture, has a major effect on the quantity of CAMP factor produced.

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A CORYNEBACTERIAL AGENT WHICH PROTECTS RUMINANT ERYTHROCYTES AGAINST STAPHYLOCOCCAL β TOXIN

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It is known that substances produced by *Str. agalactiae* and certain other micro-organisms may potentiate the action of staphylococcal beta toxin upon the washed red blood cells of the sheep and ox (Christie *et al.*, 1944; Munch-Petersen *et al.*, 1945); but that red cells may be protected against the β toxin by bacterial substances has apparently not been described before.

Protection of ox red cells against staphylococcal β toxin was first observed during routine examination of blood agar plates sown from a quarter-milk sample from a cow with a mixed infection, comprising a staphylococcus which produced β toxin, a corynebacterium and a non-Group B streptococcus. The phenomenon was subsequently observed during examination of the milk of six other cows with mixed infections by the staphylococcus and the corynebacterium.

The plates contained about 12 ml. nutrient agar to which were added 0·6 ml. defibrinated ox blood and 1 ml. of milk diluted 1 : 1,000 with saline. The ox blood was known to contain only a negligible quantity of staphylococcal β antitoxin and no α antitoxin. The phenomenon became quite clear only after incubation for 48 hours at 37° C.; the darkened zone which characteristically surrounds surface colonies of a staphylococcus producing β toxin was flattened on the side opposed to corynebacterium colonies which were nearer than about 10 mm. (Figs. 1 and 2).

In this communication we describe the phenomenon, together with some properties of the "agent." It is produced by a corynebacterium apparently identical with *C. flavidum*, the characteristics of which are given in an Appendix.

Materials and Methods.

Staphylococcal β toxin was prepared from *Staphylococcus* 32A (Bryce and Rountree, 1936), which produces β toxin only. It was sown in shallow layers of nutrient broth containing 0·1 p.c. agar and 0·1 p.c. glucose, and was incubated at 37° C. for three days in an atmosphere of 20 p.c. oxygen and 80 p.c. carbon dioxide. The supernatant, collected after centrifugation, was preserved by the addition of merthiolate to give a final concentration of 0·02 p.c., and stored at 4° C.

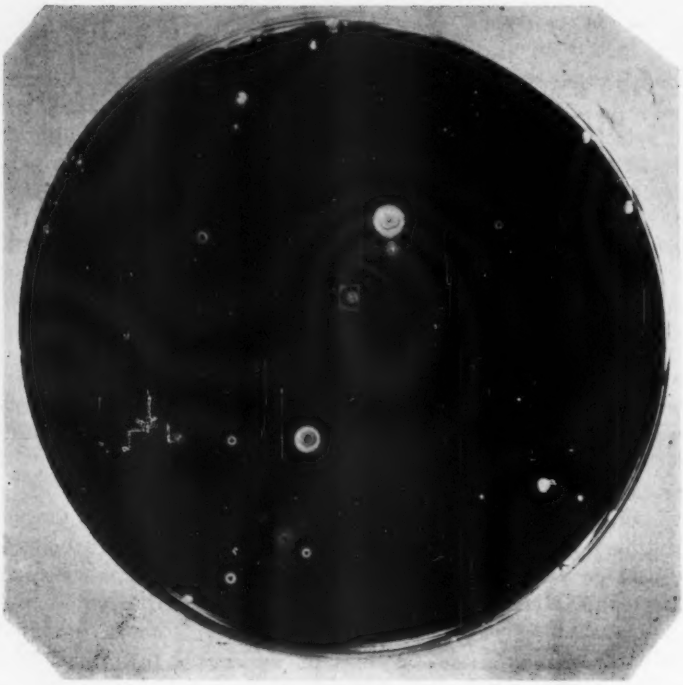

Fig. 1. Influence of neighbouring colonies of the corynebacterium upon ox red cells exposed to the action of staphylococcal β toxin. Part of agar plate, containing 5 p.c. ox blood, sown with 1 ml. milk diluted 10^{-3} and incubated for two days at 37° C.

The large colonies are due to staphylococci producing both α toxin (inner zone of complete haemolysis) and β toxin (outer zone of partial haemolysis). The small colonies with a narrow zone of complete haemolysis are corynebacteria. The β toxin zone is inhibited near corynebacterial colonies marked C. (×1.)

EXPERIMENTAL.

Some properties of the agent. It seemed likely that a diffusible substance capable of protecting the red cells against β haemolysin was produced during the growth of the corynebacterium colonies on agar. In support of this, filtrates of broth cultures through Seitz E.K. discs were also shown to prevent development of the β zone; the minimum effective dose of filtrate added to 12 ml. ox blood agar was 0·5 ml. The pH of the filtrate was originally 5·2, but became 4·0 after storage for six months, without loss of activity. Its activity was destroyed completely at 55° C. and by boiling, and was not restored by heating at 65° C. after boiling, as occurs with staphylococcal toxin (Landsteiner and von Rauchenbichler, 1909). Potency remained constant for six months at 5° C. but

decreased thereafter. Prolonged maintenance of cultures on laboratory media resulted in gradual decrease in capacity to produce the agent.

In doses of 1 ml. it was non-toxic to guinea-pigs when injected intraperitoneally, whether used at the original pH or adjusted to 7·0.

Fig. 2. Plate, after cooling at 4° C. for 48 hours to complete the haemolysis by β toxin, and after re-incubation at 37° C. for 24 hours. The inhibiting influence of the corynebacterium colony (C) upon completion of β haemolysis is demonstrated. Secondary zones of partial haemolysis due to diffusion of staphylococcal β toxin are present. The three non-haemolytic colonies are non-Group B streptococci, which exerted no obvious influence on the other colonies. The striations are due to scratches in the glass dish. (×10.)

Many unsuccessful attempts were made to obtain the active principle in a relatively pure form; the methods included concentration by freezing the filtrate at —8° C. to —20° C., or by evaporation under partial vacuum, and precipitation with absolute ethyl alcohol below —5° C., with and without buffer salts. The most active alcohol precipitate had a potency of the order of only 0·02 p.c. of the original filtrate.

Estimation of the activity. This was carried out by comparing the haemolytic effect of β toxin upon untreated cells and cells treated with the agent. In one series the amount of agent, as filtrate, was kept constant and that of β toxin was varied in serial doubling dilutions; in the other series the toxin volume was constant. After one hour at 37° C. washed sheep red cells were added and incubation was continued for another one hour. The HD_{50} of β toxin thus determined on cells previously treated with the agent was about four times that determined on untreated cells; the two tests yielded similar results.

Inhibition of the effect of β toxin upon red cell fragility. Normal ox red cells with a fragility end-point at 0·42 p.c. saline were mixed with 1 ml. of the

filtrate and were kept at 37° C. for various periods; 1 ml. of β toxin was th added, incubation was continued for 2 hours and fragility was tested. T agent prevented increase of fragility by β toxin if permitted to act upon t cells for at least 5 hours.

Effect upon red cell morphology. Observations were made on washed and sheep red cells acted on by (1) the agent only, (2) the β toxin only, a (3) the agent, followed by β toxin for varying periods of time up to 24 hou The mean diameter of the red cells acted on by the agent for 6 hours or mor whether followed by exposure to β toxin or not, was up to 60 p.c. greater tha that of normal cells or of cells exposed to β toxin (Figs. 3 and 4). Vita staining procedures failed to reveal any changes in the cells, but by phas contrast they appeared more opaque (Fig. 4).

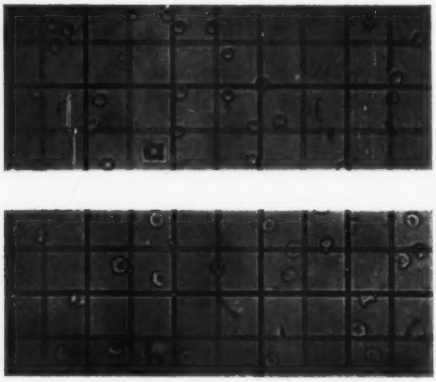

Fig. 3.

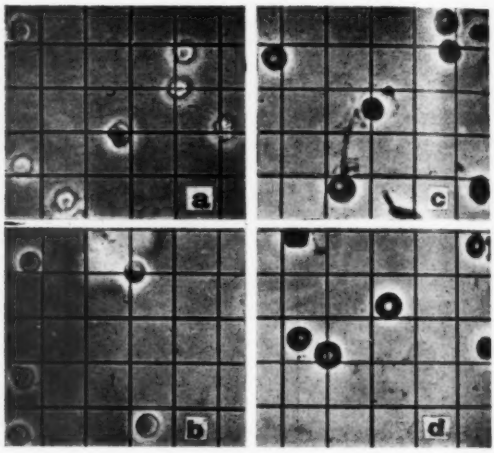

Fig. 4.

Fig. 3. Effect of filtrate of the corynebacterium upon sheep red cells. As for Fig. 4.

(a) Control suspension.
(b) Exposed to corynebacterial filtrate for 12 hours at 37° C. The cells are much larger, but generally retain their disc-like shape or are at most cup-shaped.

Unstained. Direct illumination. Haemocytometer cell.

Fig. 4. Effect of filtrate of the corynebacterium upon sheep red cells. Thrice-washed cells from oxalated blood were finally suspended in 0·85 p.c. sodium chloride before treatment.

(a) Control suspension.
(b) Exposed to staphylococcal β toxin for 12 hours at 37° C. and then for 2 hours at room temperature (20° C.). There is little change except loss of crenation.
(c) Exposed to corynebacterial filtrate ("agent") for 12 hours at 37° C. and then for 2 hours at 20° C. The cells have increased in diameter and are more opaque, but are still biconcave.
(d) Exposed to corynebacterial filtrate for 12 hours at 37° C., then staphylococcal β toxin for 3 hours at 37° C. and then for 1 hour at 20° C. Appearance similar to (c).

Unstained. Phase contrast. Haemocytometer cell.

Adsorption upon red cells. Washed sheep and ox red cells were treated with the filtrate, dilute and undilute, at 20° to 22° C., and at 37° C., for 6 and 24 hours, respectively. The red cells were then separated from the filtrate and washed thrice with saline. The centrifugate, the washed red cells and the wash saline were then individually mixed with agar; washed untreated ox red cells were added where required, and treated red cells were added direct in amounts corresponding to 5 p.c. of defibrinated blood; plates were then poured. The indicator strain producing β toxin was sown on the surface of the dried plates and incubation for 2 days at 37° C. followed; examination for β zones was made after another 24 hours at 5° C. It was found that (1) the protective agent was adsorbed from the filtrate by the red cells; (2) the treated red cells remained resistant to β toxin even after two washings, and (3) the wash saline was without activity. It is therefore concluded that the agent is firmly adsorbed to the red cells.

Failure to combine with or to inactivate β toxin. Two series of tubes were set up; one contained blood serum, from a cow known to have a high titre of staphylococcal β antitoxin, arranged in serial doubling dilutions; the other contained the Seitz filtrate of the agent, also in serial doubling dilutions. Staphylococcal β toxin in constant amounts was added to all tubes. The volumes were equalized with saline.

After incubation for one hour at 37° C., washed sheep red cells were added to give a final concentration of 10 p.c.; after reincubation for one hour at 37° C. the red cells remained quite unchanged. They were still unchanged in the series containing β antitoxin after being kept for a further 24 hours at 5° C., i.e., the serum had protected the red cells against the β toxin to the usual titre, but the filtrate had not protected. Evidently the protective agent does not inactivate β toxin.

In a control tube containing only agent and red cells, there was no haemolysis, i.e., the haemolysin produced by the corynebacterium on agar was not present in detectable amounts in the filtrate.

A staphylococcus known to produce β toxin was grown in nutrient broth and in broth with the following additions:

(1) fresh blood serum from cow A having a high titre of staphylococcal β antitoxin, (2) fresh blood serum from cow B having a high titre of staphylococcal β antitoxin, (3) fresh blood serum from cow C having no titre of staphylococcal β antitoxin, (4) Seitz filtrate containing corynebacterial agent, (5) Seitz filtrate containing corynebacterial agent plus serum from cow A, (6) Seitz filtrate containing corynebacterial agent plus serum from cow B, and (7) Seitz filtrate containing corynebacterial agent plus serum from cow C.

The final concentration of serum was 5 p.c. in each tube.

Washed sheep red cells were added to a final concentration of 5 p.c. and the tubes were incubated at 37° C. After 24 and 48 hours, "spot" inoculations were made from each tube on agar plates containing 5 p.c. washed sheep red cells. There was no difference in the appearance of the darkened ring around the colonies deriving from each of these tubes.

Growth was equally good in all tubes; the red cells in the control tube were lysed to about 50 p.c. in 48 hours, those in (5) and (6) to about 25 p.c. Refrigeration for 24 hours resulted in complete lysis in control and (3); slight lysis in (5), (6) and (7); very slight in (1) and (2); no visible change in (4).

Thus, none of the additions prevented the development of the β toxin, but the serum from cows A and B as well as the corynebacterial agent protected the red cells, although not to the same extent. Normal growth of the staphylococcus was not prevented, as shown by the subculturing on agar from the tubes.

Necessity for prior contact with red cells. When staphylococcal β toxin was added to a tube containing 10 p.c. washed sheep red cells in saline or broth up to 5 hours before the agent, lysis did not occur after incubation for 2 hours at 37° C., but did after subsequent refrigeration for 18 hours; in other words, the β toxin behaved normally and had not been inactivated or displaced by the agent. When the addition of the corynebacterial agent to the red cells preceded that of the toxin under similar conditions, the haemolytic effect of the toxin was entirely prevented.

DISCUSSION.

It has been shown that the corynebacterial agent does not inactivate or combine with β toxin, at least in the bulk phase, but that it combines with or is adsorbed by the erythrocytes, and, furthermore, that this is associated with an increase in size of the cells and complete resistance to the action of β toxin. The nature of the increase in size has not been determined; it does not appear to be an osmotic effect associated with change of permeability of the cell membrane because the cells retain more or less the disc-like shape and do not become distended spheres; it appears to be associated with an actual increase in surface area unrelated to turgidity. The agent is distinct from the haemolysin and from toxin.

APPENDIX.

Description of the corynebacterium.

The characteristics agreed fairly well with those of *C. flavidum* (Morse), Bergey *et al.* (1939). Strains (total 26) from four different cows were tested. A further 5 cultures from another cow were subjected to only some of the tests. In view of the brief description in Bergeys' Manual, we have appended our details:

Morphology (24-hour cultures of original isolates in broth at 37° C.).

Pleomorphic rods, mostly about 0·25 to 0·5μ by 2 to 3μ; many coccoid forms and long filaments which increase with age of the culture, Gram-positive; non-motile, not acid-fast, no spores; fairly large metachromatic granules. Many cells are club-shaped, with the arrangement typical of corynebacteria.

Cultural characteristics.

Gelatin. Good growth throughout stab; no liquefaction by 8 strains; 11 strains produced visible liquefaction in 2 weeks, increasing with age to 5 weeks.

Agar plates (poured). Growth only visible at 48 hours; on the fifth day deep and surface colonies are circular, 1-1·4 mm. in diameter, white, smooth, entire, "heaped-up" appearance; no crystals; no odour; no digestion.

Agar slants. Colonies become visible in 48 hours. In 3 days discrete, white, entire, heaped-up; slightly moist, not confluent. At 5 days: profuse, slightly spreading, with erose edge. No crystals, odour or digestion.

Agar shake cultures. Excellent growth on surface and throughout the medium; similar to agar plates.

Blood agar (ox). Slight haemolysis around deep but not surface colonies; growth otherwise as on agar.

Citrate agar. Fair growth, as for agar. No change in medium.

Broth (with and without serum or dextrose). Growth visible already after 24 hours; excellent growth, clear supernatant, finely granular white sediment, easily shaken up. No odour; no crystals. No pellicle or growth on surface.

Litmus milk. No change in 5 days, but may be reduced in 3 weeks; colour returns on shaking (8 strains): 11 developed no visible change.

Potato. Very doubtful growth; survived 3 weeks, no change in medium, no odour.

Loeffler's serum. Moderate growth, slightly raised, low convex, erose edge, opaque. No odour, no liquefaction, no digestion.

Biochemical reactions.

All strains—total 31—excreted the agent equally quickly and to the same strength as shown when tested with a staphylococcus producing β toxin grown on ox or sheep blood agar.

Catalase. Positive.

Hydrogen sulphide. None by 6, some by 13 strains.

Methylene blue. Reduced by 8, not by 11 strains.

Voges-Proskauer test. Negative.

Methyl-red test. One strongly positive, one weakly positive, 17 negative.

Indole. Negative.

Ammonia. Negative.

Nitrate. Not reduced.

Pigment. 8 strains produced yellowish-green pigment soluble in the medium.

Fermentation reactions[1].

Arabinose, xylose and rhamnose (11 strains tested), no acid; glucose (19 tested), acid; fructose (16 tested), acid; mannose (16 tested), acid; galactose (31 tested), 15 acid, 16 no acid; saccharose (14 tested), one slightly acid, 13 no acid; maltose (26 tested), 15 acid, 11 no acid; lactose (14 tested), 13 acid, 1 no acid; trehalose (16 tested), acid; raffinose (11 tested), no acid; starch (16 tested), 12 acid, 4 no acid; inulin (11 tested), no acid;

[1]Barrett *et al.* (1942) point out that fermentation of certain carbohydrates depends on the composition of the culture medium and the indicator used.

dextrin (16 tested), acid; glycogen (16 tested), acid; glycerol (23 tested), 7 acid, 16 no acid; erythritol (11 tested), no acid; adonitol (11 tested), no acid; mannitol (14 tested), 2 acid, 12 no acid; dulcitol (11 tested), no acid; sorbitol (11 tested), no acid; salicin (11 tested), no acid; inositol (11 tested), no acid; amygdalin (11 tested), no acid. Gas not produced from any carbohydrate.

Of 12 strains tested in peptone water plus inactivated ox serum with maltose, all produced acid on the first day. From the second day onwards the acid reaction gradually disappeared, completely by the 8th day with 7 strains, whereas the acid reaction remained with 5 strains. The same 12 strains tested simultaneously on Spray's (fermentation base) agar slopes with maltose did not yield acid. The same 12 strains yielded acid in 6 days from galactose incorporated in Spray's agar slopes, but not in 8 days in peptone water plus inactivated ox serum, although growth was good.

Pathogenicity.

A 24-hour broth culture injected intramuscularly was lethal to 4 guinea pigs in one to three days (1 ml.) and to 7 out of 8 mice in 6 to 13 days (0·5 ml.).

Thermal death point.

60° C. for 5 minutes, 24-hour broth cultures.

SUMMARY.

Certain slightly haemolytic diphtheroid bacilli occasionally found in aseptically-drawn cow's milk excrete a substance which protects sheep and ox red cells from staphylococcal β toxin, with which, however, it does not combine.

The agent is filter-passing, thermolabile, and apparently distinct from the haemolysin.

A description is given of the diphtheroid bacillus responsible; it is probably closely related to, if not identical with, *C. flavidum* (Morse) Bergey *et al.*

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STUDIES IN EXPERIMENTAL IMMUNOLOGY OF INFLUENZA

X. PASSIVE IMMUNITY AND ITS ENHANCEMENT

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The experimental evidence contained in the previous papers of this series is consistent with the view that the degree of resistance to influenza virus infection in mice was determined by the amount of specific antibody present in the respiratory tract. The need of postulating some "cellular" type of immunity did not arise. Recognition of the fact that only a small fraction of the antibody produced is *immunologically active* (capable of preventing infection) led to a detailed study of the factors governing the distribution of antibody between the circulation and the site of possible infection, and to a search for agents capable of shifting this distribution in favour of the respiratory tract, i.e., enhancing active immunity.

The present paper describes experiments performed on mice immunized passively against influenza. This approach enabled us to study protection in animals which had no previous experience with the challenging virus, and thus made it possible to separate strictly the effects of the presence of antibody from the effects of past production of antibody, i.e., sensitization. Special interest was taken in the distribution of antibody introduced passively into the organism by different routes. These experiments served as a baseline for the testing of pathotopic potentiation of passive immunity, the investigation of which is described in the second half of the paper.

MATERIALS AND METHODS.

Normal saline. 9.00 gm. NaCl in 1,000 ml. distilled water.

Red blood cells. Venous blood of adult fowls was collected in Alsever's solution. The cells were washed in two changes of normal saline, packed and stored at 4° C. No cells older than 36 hours were used in the experiments.

Virus. The MEL strain of influenza A virus was grown allantoically in 11-day-old White Leghorn eggs. Allantoic fluids, harvested 42 hours after infection with approximately 100 ID_{50} of glycerolated seed, were pooled and stored at 4° C. No fluid older than seven days was used in the experiments.

Antisera. A single batch of MEL-virus-containing allantoic fluid served as antigen in the preparation of antisera. The original pool had an infectivity titre of $10^{8.3}$ ID_{50}/0·10 ml., and a haemagglutinin titre of 800 against an equal volume of 1 p.c. red cells. It was used either in the active state or after inactivation overnight by 0·05 p.c. formaldehyde.

Mice received two doses of 0·30 ml. of the formolized vaccine by the peritoneal route at an interval of six days. The serum was collected ten days after the second injection.

Rabbits were inoculated intravenously with 1·5 ml. of killed vaccine, and received two further doses of the same size at five-day intervals. The animals were bled five days after the last injection.

Ferrets were given 0·25 ml. of active MEL virus by the nasal route, and bled out twelve days after infection.

Fowls received 1·5 ml. of live virus intravenously, and were bled six days after immunization.

Sera within each group were pooled, inactivated by heating at 62° C. for 15 minutes, and stored in the frozen state at —10° C. without any chemical preservative. Immediately before use the required volumes were thawed, lightly spun to remove any precipitate, and tested for their antihaemagglutinin content. It was found that the titres have remained unchanged over the period of this study.

Titration of antihaemagglutinin. Falling two-fold dilutions of the sample were made in 0·25 ml. volumes of 0·5 p.c. fowl red blood cells in normal saline. One standard drop (approximately 0·03 ml.) of saline containing exactly 5 agglutinating doses of active MEL virus was then added to each tube, the racks shaken, and the pattern of settled cells read after 30 minutes' standing at room temperature. Partial agglutination was taken as the endpoint, and the titre is the reciprocal of the endpoint-dilution.

Assessment of immunity. The protection-test devised earlier in the course of these studies was used without modification. For full details of the technique and a critical discussion see the original paper (Fazekas de St.Groth and Donnelley, 1950b).

EXPERIMENTS.

The fate of antibody introduced by different routes.

The *first set of experiments* served to determine what portion of antibody found its way into the circulation after various amounts of homologous and heterologous antisera were administered by different routes.

Mice of both sexes weighing 21-24 gm. (between the age of 4½ and 6 weeks) were given 0·50, 0·15 or 0·05 ml. inocula intravenously, intraperitoneally or subcutaneously, or 0·05 ml. volumes instilled intranasally. Undiluted sera of mice, rabbits, ferrets and fowls were used; the specific antibody titres of the pools will be given in the Tables below. Three hours after immunization serum samples were taken from groups of ten mice and titrated individually for antibody against active MEL virus. Table 1 gives the results.

In computing the "percentage of antibody in serum" the average plasma volume of a 21-24 gm. mouse was taken as 0·90 ml., which represents 22 p.c. of the total extracellular fluid. It was further assumed that the increase of this volume by that of the inoculum is only transient, since the healthy organism will maintain its homeostasis by rapidly excreting excess water or electrolytes.

Of the several interesting points emerging from the analysis of this Table, the one with immediate bearing on subsequent experiments is the finding that after both intravenous and intraperitoneal passive immunization large amounts of antibody were found in the circulation three hours after administration.

Indeed, the percentage was higher than would be expected if there were a ready equilibration between the two compartments of body fluid. Since there was no appreciable difference between the two routes, in the later experiments we felt justified in using the peritoneal route as the equivalent and instead of the more laborious intravenous injections. It should also be noted that after intraperitoneal and intravenous inoculation the final result was independent both of the amount of antibody given and of the volume in which it was contained.

TABLE 1.

Serum antibody titres of mice after passive immunization.
(Blood samples taken three hours after immunization.)

Antibody used			Route of administration							
			Intravenous		Intraperitoneal		Subcutaneous		Intranasal	
Source	Titre	Volume given	Titre	p.c.	Titre	p.c.	Titre	p.c.	Titre	p.c.
Mouse	240	0·50	41	31	35	26	19	14		
		0·15	11·6	26	10·7	24	—			
		0·05	3·7	28	3·3	25	—		—	
Ferret	4800	0·50	695	26	641	24	455	17		
		0·15	256	29	238	27	19·1	2		
		0·05	70	26	75	28	3·4	1	—	
Rabbit	1200	0·50	192	29	181	27	93	14		
		0·15	59	30	50	25	9·6	5		
		0·05	17·0	26	19·6	29	—		—	
Fowl	3200	0·50	548	31	304	17	162	9		
		0·15	128	24	114	21	10·5	2		
		0·05	44	25	25	14	—		—	

Each figure gives the geometric mean titre of a group of ten mice. —=<3.
"Percentage of antibody in serum" was calculated by the formula:

$$\frac{100 \times \text{titre of sample} \times \text{circulating plasma volume}}{\text{titre of immunizing inoculum} \times \text{volume injected}}.$$

This certainly was not the case after subcutaneous introduction of antisera. Larger proportion of antibody found its way into the circulation when the inoculum was of comparatively larger volume (0·50 ml., as compared with 0·05 ml.). The absolute amount of antibody injected did not seem to play any part in this distribution as the gradation was almost identical in the case of ferret and rabbit sera, though there was a fourfold difference between the concentration of antibody in them. Further, the percentual recovery after 0·50 ml. ferret or mouse serum was for all practical purposes the same though the latter contained twenty times less antibody in the same volume. The other characteristic of the subcutaneous route was that even the highest amount of antibody appearing in the circulation was but a small fraction of the amount injected. In a similar but more pronounced manner did this hold for the nasal route. After this type of passive immunization no detectable quantity of antibody appeared in the serum. In this instance, however, the range over which tests could be made was rather restricted owing to the size of the inoculum which can be given by the nasal route.

In the *second experiment* the effect of time on serum titres was investigated.

Large groups of mice were immunized passively; materials were the same as in the first experiment. Serum samples were taken from groups of ten animals —either by bleeding from the tail vein or by exsanguination through the severed art. subclavia—1 and 3 hours after immunization, and then ten times during the first fortnight. Antibody titres were determined individually on the day of sampling. To ensure uniformity, at least ten sera from the previous day were included in each test. The geometric mean titre of these replicate titrations differed from the original by less than 11 p.c. Yet, since six out of the eight differing sets of sera showed lower average titres on re-testing, it is not impossible that the difference was due to deterioration on storage at 4° C. for 24 hours between titrations. This being the case, any correction might have introduced a virtual systematic error. The values presented in Table 2 are the original readings, and might be thus 11 p.c. (0·16 $\log_{10}$ units) incorrect due to daily variations in conditions of the test. A remarkable feature of these experiments was the impressive uniformity of readings within each group of ten animals; indeed, to one used to the wide scatter of antibody titres after active immunization, it came as a pleasant surprise, making a more exact evaluation possible.

TABLE 2.

The fate of passive antibody in the mouse.

	Antibody administered				Antihaemagglutinin titre of mouse serum after												
		Volume			Hours			Days									
Source	Titre	given	Route	Dilution	—1	1	3	1	2	3	4	5	7	8	10	12	14
Mouse	240	0·20	i.v.	1:1	—	30	18	10	8	6	5	4	<4	—	—	—	—
		0·20	i.p.	1:1	—	10	14	10	9	7	6	5	<4	—	—	—	—
		0·20	s.c.	1:1	—	—	5	9	6	5	5	5	<5	<4	—	—	—
		0·05	i.n.	1:1	—	—	—	—	—	—	—	—	—	—	—	—	—
Ferret	4800	0·20	i.v.	1:1	—	438	307	238	153	80	51	36	27	23	18	14	11
				1:4	—	80	73	46	40	25	16	10	8	6	<4	—	—
		0·20	i.p.	1:1	—	238	295	219	141	73	51	37	30	30	18	16	15
				1:4	—	38	50	46	33	19	11	9	8	7	5	—	—
		0·20	s.c.	1:1	—	9	34	117	80	64	40	37	34	23	19	16	14
				1:4	—	—	6	25	20	18	11	9	9	7	<5	<4	—
		0·05	i.n.	1:1	—	—	—	<4	6	5	<4	—	—	—	—	—	—
Rabbit	1200	0·20	i.v.	1:1	—	117	76	36	28	19	11	8	6	4	—	—	—
				1:4	—	32	19	10	7	5	4	—	—	—	—	—	—
		0·20	i.p.	1:1	—	59	64	34	23	16	11	9	6	5	—	—	—
				1:4	—	10	17	9	8	5	<4	—	—	—	—	—	—
		0·20	s.c.	1:1	—	5	10	30	20	18	14	9	7	5	<4	—	—
				1:4	—	—	—	6	6	4	<4	—	—	—	—	—	—
		0·05	i.n.	1:1	—	—	—	—	<4	—	—	—	—	—	—	—	—
Fowl	3200	0·20	i.v.	1:1	—	318	238	40	10	7	4	—	—	—	—	—	—
				1:4	—	50	46	16	5	—	—	—	—	—	—	—	—
		0·20	i.p.	1:1	—	119	77	25	8	5	<4	—	—	—	—	—	—
				1:4	—	18	20	8	4	<4	—	—	—	—	—	—	—
		0·20	s.c.	1:1	—	7	17	5	<4	—	—	—	—	—	—	—	—
				1:4	—	—	—	—	—	—	—	—	—	—	—	—	—
		0·05	i.n.	1:1	—	—	—	—	—	—	—	—	—	—	—	—	—

Each figure gives the geometric mean titre of a group of ten mice. —=<3.

The behaviour of the three mammalian antisera given passively to mice was essentially identical, but the fowl serum disappeared much more rapidly.

The difference between the intravenous and intraperitoneal routes on one hand, and the subcutaneous and intranasal on the other, which was so definite in the first experiment (cf. Table 1), here appears to be relative, and a function of time. The antibody curve after intranasal administration of antisera is still strikingly different from the others, although the difference might be only quantitative, as suggested by the behaviour of ferret sera.

There is a phase which extends over the first 48 hours after administration of antiserum, and is characterized by the distribution of foreign antibody in the recipient organism. During this phase the serum titre depends on the route of passive immunization. After intravenous immunization there is a precipitate drop during the first day—antibody, originally all present in the vascular bed, enters the non-circulating compartment of extracellular fluid. After intraperitoneal injection the maximal serum titres are not reached immediately—in the case of mammalian sera it seems to take about three hours. At this time closely similar serum antibody titres are found for both intravenous and peritoneal passive immunization, and the further behaviour of the curves is also identical. After subcutaneous administration the escape of antibody from the site of injection is considerably slower; however, by the end of the second day the curves approach those of the intravenous and intraperitoneal routes, and overlap for the rest of their course. Only very small quantities of antibody reach the circulation after intranasal instillation, with a maximum—as far as can be guessed from the scanty data—on the second day.

The next phase, that of steady elimination, starts about 48 hours after immunization. The differences during this period are not between the various routes of administration but between the different types of sera injected. The order of increasing rate of elimination gives the series: mouse <rabbit <ferret $\leqslant$fowl. The slopes of the curves are independent of the amount of antibody given (see Table 2), and fall on a straight line when log concentration is plotted against time (see Fig. 1).

The last phase is characterized by a slowing down in the elimination of antibody. This change in the rates is relative; it is distinguishable on all curves in Fig. 1, but in the case of fowl antibody even the flatter slope (third phase) is steeper than that of the second phase for most of the other sera.

The kinetics of the whole process are far from simple, but it should be noted that in the above experiments the presence of antibody directed against the foreign serum did not complicate the picture. This became evident from some additional tests in which we could not detect any precipitin against the heterologous sera in mice immunized 12 or 14 days previously. Furthermore, the fact that antibody did *not* disappear *faster* in the third phase would suggest the same. The strongest, though indirect, argument is the finding that in animals successfully immunized against a heterologous serum the disappearance of passive anti-

body gave curves entirely dissimilar to those of Fig. 1. As much of these results as is relevant to the understanding of passive immunity against influenza in mice will be discussed at the end of this paper; the rest, which bears on the more general problem of passive immunization, will form the subject of a separate communication.

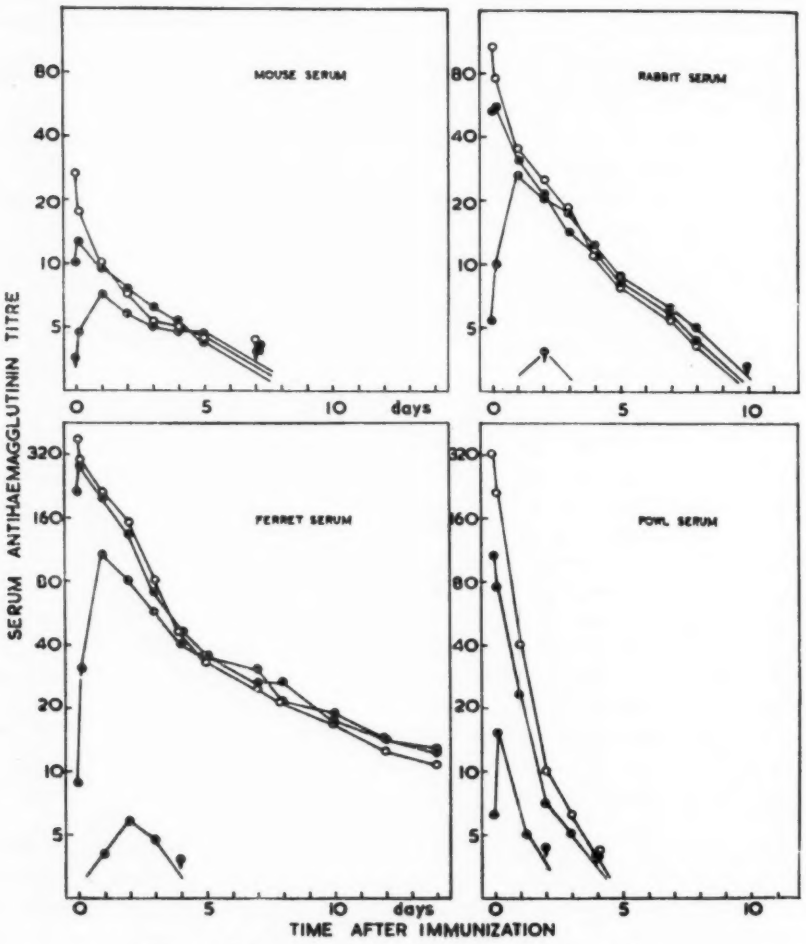

Fig. 1. *Serum antibody titres after passive immunization.*

Routes of immunization:
—O—O— = intravenous (0·20 ml.).
—●—●— = intraperitoneal (0·20 ml.).
—⊙—⊙— = subcutaneous (0·20 ml.).
—②—②— = intranasal (0·05 ml.).

Antibody titres of original inocula:
Mouse serum: 240/0·25 ml.
Rabbit serum: 1200/0·25 ml.
Ferret serum: 4800/0·25 ml.
Fowl serum: 3200/0·25 ml.

The protective effect of passive immunization.

With the results of the preliminary tests in hand, a single large experiment was set out both to show the protection afforded by passive immunization, and to find out whether or not it could be enhanced by the use of pathotopic adjuvants. Ferret anti-MEL serum was used in a dilution to give titres comparable to those found in mice after active immunization with a potent vaccine. This experiment did not work out as expected: the presumably highly protected animals died almost as readily as untreated controls, and the adjuvated groups were not significantly different from the rest.

This failure pointed forcibly to some misconception in our planning, and made a more extensive, systematic study of passive immunity imperative. The following group of experiments served this purpose.

In the first of these tests the protective effect of passive immunization by the peritoneal route was investigated. Of the four anti-MEL sera studied in the previous experiments only the three mammalian sera (ferret, rabbit, mouse) were used consistently; fowl serum, because of its too rapid elimination, was omitted from some of the tests. The effectiveness of foreign antibody was evaluated in relation to antihaemagglutinin titres. The mouse serum (original titre 240) was diluted in fourfold steps, giving a series with titres of 240, 60, 15 and 3·75. The ferret and rabbit sera were both made up to titres of 960, 240, 60 and 15, thus providing series which had three common steps each with the mouse serum dilutions.

Groups of 30 five-weeks-old mice received 0·40 ml. of the different serum dilutions intraperitoneally. A group of 40 control animals was given the same volume of saline. Two days after passive immunization (this interval was chosen to allow adequate distribution through the tissues; cf. Table 2) the 30 mice of each group were divided into six sub-groups of 5, and challenged by the nasal route with dilutions of active MEL virus ranging from 10^{0} to 10^{-5}. The eight sub-groups of the control animals received inocula ranging from 10^{0} to 10^{-7}. The infectivity test was conducted as described earlier (Fazekas de St.Groth and Donnelley, 1950b); the "protection values" show how many times higher the infective dose had to be to cause standard symptoms, as determined in the set of untreated control mice. Ten mice of the groups receiving passive antibody titering 240 were bled from their caudal veins on the day of challenging. In keeping with expectations, their serum anti-haemagglutinin titres ranged between 13·2 and 16·8, i.e., were of the same order irrespective of the source of antibody administered.

The results of this experiment (Table 3) could not have been predicted from our previous findings. First, intraperitoneally injected mouse serum was approximately thirty times as effective as the same amount of either ferret or rabbit serum. Secondly, the protection of mice passively immunized with heterologous sera was on the average only one-fiftieth of what would have been afforded by similar serum titres resulting from active immunization by the peritoneal route.

TABLE 3.

Protective effect of passive immunization by the peritoneal route.

(The mice were challenged with active MEL virus 48 hours after the inoculation of anti-MEL sera.)

Source	Antiserum Volume given	Titre	Infectivity test ΣL	d_x	Log P	Protection (P)
Ferret	0·40	960	31·25	7·80	2·91	824
		240	33·42	5·63	2·10	126
		60	36·65	2·40	0·86	7
		15	38·76	0·29	0·11	1
Rabbit	0·40	960	28·46	10·59	3·94	8,700
		240	31·46	7·59	2·83	676
		60	36·37	2·68	1·00	10
		15	39·24	—0·19	—0·07	1
Mouse	0·40	240	29·31	9·47	3·63	4,270
		60	31·90	7·15	2·67	468
		15	34·17	4·88	1·82	66
		3·8	38·22	0·83	0·31	2
Saline (control)	0·40	—	39·05	$d_{10}=$ 2·68	0·00	1

These findings account for our failure to get satisfactory protection with peritoneally administered ferret serum, but do no more than hint at the underlying mechanism. According to our hypothesis of antibody action in experimental influenza, only that fraction of antibody is instrumental in preventing infection which meets and neutralizes the virus in the respiratory tract (Fazekas de St.Groth and Donnelley, 1950b). From this reasoning it would follow that heterologous mammalian sera, when injected peritoneally, do not penetrate into the respiratory tract as readily as does homologous antiserum, be it given passively or produced by the animal itself after active immunization. Formal proof of this thesis is required at two points, both open to direct experimental testing: (a) it should be shown that identical amounts of the different antibodies have the same neutralizing power when mixed with the virus *in vitro,* or when used for passive immunization by the *nasal* route; and (b) that after parenteral administration of identical amounts of heterologous and homologous antisera the bronchial washings of mice contain in fact more of the homologous antibody. Both of these assumptions were tested and confirmed by the following experiments.

For the straight infectivity test 0·20 ml. of active MEL virus dilutions in horse serum saline were incubated at 0° C. for 60 minutes with equal volumes of antiserum dilutions. Then 0·05 ml. volumes of these mixtures were given to groups of 5 mice by the nasal route. Death times were recorded, and all sur-

vivors autopsied on the seventh day. The effect is assessed in terms of the amount of virus neutralized ("protection value"), as has been done throughout in the standard protection-test. The results are presented graphically in Fig. 2.

It can be said that, antihaemagglutinin titre for antihaemagglutinin titre, the four antisera have practically the same neutralizing power against the infectivity of their common antigen. It is of interest, however, that the slopes of the neutralization curves (straight lines over the range tested when log concentration is plotted against the log of virus neutralized) are not quite identical, and all of them differ significantly from unity. This fact is pointed out not so much for its relevance to the theme of the present study, but because it will be dealt with in a later paper on the kinetics of antigen-antibody combination.

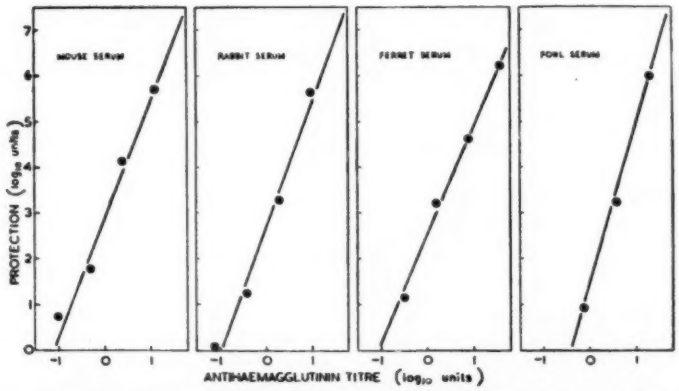

Fig. 2. *The correlation between neutralizing antibody and antihaemagglutinin in various antisera.* (For description of the test, see text.)

The next experiment, passive immunization by the nasal route, is essentially a simple neutralization test from which the pre-incubation *in vitro* of the antigen-antibody mixture has been omitted. Groups of 30 mice were given different dilutions of anti-MEL sera; 0·05 ml. volumes were instilled intranasally. Again, the titre of 240 was taken as a starting point. After an interval of three hours each group was challenged with active MEL virus, sub-groups of five mice being given dilutions from 10^0 to 10^{-5}. Control animals, inoculated with saline instead of antiserum, received virus in concentrations from 10^0 to 10^{-7}. The results, for the sake of easier comparison with Table 3, are expressed as "protection values".

The protective value of the three anti-MEL sera when given by the nasal route is essentially identical (Table 4); indeed, the picture resembles in all its main features that obtained by the orthodox neutralization test (see Fig. 2). Again, the readings would fall on straight lines when plotted on a log-log scale,

the slopes being significantly different from unity. The difference between the two sets of data is only quantitative: with previous incubation *in vitro* antibody is approximately a hundred times more effective than when used for passive respiratory immunization three hours in advance of the infective inoculum.

TABLE 4.

Protective effect of passive immunization by the nasal route.

(The mice were challenged with active MEL virus 3 hours after the inoculation of anti-MEL sera.)

Source	Antiserum Volume given	Titre	Infectivity test ΣL	Infectivity test d_x	Log P	Protection (P)
Ferret	0·05	960	25·04	12·78	5·64	437,000
		240	27·00	10·82	4·76	57,500
		60	29·43	8·39	3·69	4,900
		15	32·35	5·47	2·41	257
Rabbit	0·05	960	23·97	13·85	6·11	1,290,000
		240	26·60	11·22	4·95	89,100
		60	31·27	6·55	2·88	759
		15	33·20	4·62	2·03	107
Mouse	0·05	240	26·11	11·71	5·17	148,000
		60	28·84	8·98	3·95	8,910
		15	31·66	6·16	2·71	523
		3·8	34·04	3·80	1·67	47
Saline (control)	0·05	—	37·82	$d_{10}=$ 2·27	0·00	1

The last two experiments demonstrated that the three antisera have comparable neutralizing and protective properties, and showed, indirectly, that the difference found between the homologous mouse serum and the two heterologous sera was genuine and not caused by some technical error. What remained to be determined was the amount of antibody which finds its way into the respiratory tract after extrarespiratory passive immunization with antisera of different origin. It is known that after parenteral vaccination roughly 1/250 of the total antibody produced can be recovered in bronchial washings (Fazekas de St.Groth and Donnelley, 1950a). Consequently, to be able to work over a range of well measurable concentrations, large amounts of antibody were injected peritoneally. Groups of mice were given 1·0 ml. inocula of ferret serum 1:1, 1:2 and 1:4; rabbit serum 1:1 and 1:2; and undiluted mouse serum. Ten mice of each group were killed 24 and 48 hours after immunization, and blood samples and bronchial washings taken. Antihaemagglutinin titres against active MEL virus were determined in all samples simultaneously on the second day.

After a preliminary test, in view of the very low antibody titres in bronchial washings, the technique had to be modified to increase its sensitivity. By using

the same volume of the sample to be titrated, but a four times weaker cell suspension (0·25 p.c.) and a four times diluted standard agglutinating dose (1·25 AD), the smallest amount of antibody that could be measured became a titre of 0·125 instead of 0·5, the limit in the standard test. Many of the endpoints were missed even by this improved method; in these cases only a limiting maximum value can be given for the ratio of serum and bronchial antibody titres.

TABLE 5.

Distribution between serum and bronchi of antibody injected by the peritoneal route.

Antibody administered		Antihaemagglutinin titre					
		After 24 hours			After 48 hours		
Source	Titre	Serum	Bronchi	Coeff.*	Serum	Bronchi	Coeff.*
Ferret serum	19200	1046	0·29	0·01	712	0·78	0·05
	9600	612	0·18	0·01	348	0·39	0·06
	4800	256	<0·13	<0·03	185	0·17	0·05
Rabbit serum	4800	214	<0·13	<0·03	152	0·20	0·07
	2400	132	<0·13	<0·05	89	<0·13	<0·08
Mouse serum	1200	68	0·17	0·12	49	0·34	0·35

*The "distribution coefficients" were calculated by the formula:

$$\frac{100 \times \text{bronchial antibody titre}}{2 \times \text{serum antibody titre}}$$

(cf. Fazekas de St.Groth and Donnelley, 1950a).

The results of Table 5 are of interest because of their negative nature. A significant point is that, though of lowest titre, it is the mouse anti-MEL serum which appeared in bronchial washings in the largest proportion. The "distribution coefficient" on the second day, 0·35, is only slightly different from that found after active immunization by parenteral routes, 0·42. The other two antisera used for passive immunization give distribution coefficients of less than 0·1 in whatever dilution they were used; a more exact estimate can be given only in the case of the very highest concentrations, it would be about 0·05, i.e., only one-seventh as great as with homologous passive immunization. This difference is highly significant, and points to some intrinsic difference in the mechanism of distribution of homologous and heterologous antibody globulin in the mouse. It also gives a satisfactory explanation for the relatively high protective effect of intraperitoneally administered mouse antisera compared with heterologous sera given by the same route.

The bronchial antibody titres increased in every instance between day 1 and 2, and this was the more remarkable as the serum antibody titres were dropping at the same time. The distribution of antibody appears thus to be a lengthy process, a finding which is in agreement with the results of Table 2.

One more experiment is pertinent at this juncture. It is concerned with the time relations of passive immunization, and further stresses the fundamental difference between the nasal and extrarespiratory routes.

Groups of mice were passively immunized 2, 1 days or 1 hour before, or 1 day after challenging. The only antiserum used in this experiment was a mouse serum pool with an anti-MEL titre of 280. Dilutions of 1:1, 1:5 and 1:25 were made up in normal saline and instilled nasally in 0·05 ml. volumes or injected peritoneally in 0·20 ml. volumes. "Protection values" were determined by the standard technique, based on findings from groups of 30 mice. In Fig. 3 the log protection values are plotted against the time by which immunization preceded experimental infection.

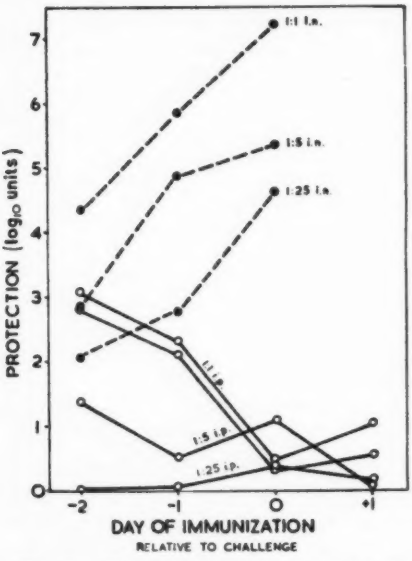

Fig. 3. *The protective effect of nasal and peritoneal passive immunization.*

Dilutions of mouse anti-MEL serum (original titre: 280/0·25 ml.) were given in volumes of 0·05 ml. intranasally (-●——●-) or 0·20 ml. intraperitoneally (-O——O-), at the times shown on the abscissa.

There is a striking difference in the trend of the intranasal and peritoneal immunity curves. Nasal passive immunization is most effective immediately before challenging, and with increasing interval there is an increasing drop in efficacy. The regression is compatible with the kinetics of a monomolecular reaction: a standard percentage is eliminated in a standard time. Following peritoneal passive immunization, the height of protection is not reached before the second day after the administration of antibody; at this time the serum antibody titres are already significantly lower than initially. The general character of the curves is independent of the amount of antibody given.

The results, besides demonstrating once more the slowness of equilibration of antibody between the circulation and the bronchial tract, have also a strong bearing on the practical aspects of passive immunization against respiratory infections. Since maximal antibody titres at the site of infection are not reached before 48 hours after the administration of antibody, it would appear futile to expect favourable results when passive immunization is followed immediately by challenging, as has been done almost without exception in the past.

Potentiation of passive immunity.

In the final set of experiments the effect of pathotopic adjuvants was tested under conditions now known to be optimal for their action.

Groups of 30 mice (5-6 weeks old) were given 0·05 ml. volumes of pathotopic adjuvants by the nasal route. The respective concentrations were as follows: KIO_4 0·2 p.c., $ZnSO_4$ 0·08 p.c., tannin 0·15 p.c., all diluted in normal saline; the control group received the diluent only. Five or six days later all groups were passively immunized. Mouse anti-MEL serum (titre 240) or rabbit anti-MEL serum (titre 480) were injected peritoneally in 0·20 ml. volumes. The double amount of heterologous serum was used to compensate for its poorer permeation of the respiratory tract (cf. Table 5). Two days after immunization all groups were challenged with graded doses of live MEL virus and the results evaluated by the standard method used in these studies. Full details of the tests are presented in Tables 6 and 7.

TABLE 6.

Pathotopic potentiation of passive immunity.
A. Homologous serum.

Treatment	Infectivity test ΣL	d_x	Log P	Protection P	Potentiation P_X/P_M
M^2	32·06	5·91	2·89	776	1
I^2M^2	26·82	11·15	5·46	288,000	363
Z^2M^2	27·12	10·85	5·31	204,000	257
T^2M^2	28·22	9·75	4·77	58,900	69
Nil (control)	37·97	$d_{10}=$ 2·045	0·00	1	

Treatments: M=mouse anti-MEL serum, 0·20 ml. peritoneally;
I=IO_4^- M/100 in normal saline, 0·05 ml. nasally;
Z=$ZnSO_4$ 0·08 p.c. in normal saline, 0·05 ml. nasally;
T=tannin 0·15 p.c. in normal saline, 0·05 ml. nasally.
The *index numbers* show the interval, in days, between consecutive treatments.

Table 6 shows that passive immunity attained by peritoneal administration of homologous antiserum can be considerably enhanced by the use of pathotopic adjuvants. The basic immunity (group "*M*"), as has been pointed out before, is lower than would be afforded by similar serum antibody titres after active immunization. On the other hand, the increase of protection values over this level in the adjuvated groups ("*I-M*", "*Z-M*", "*T-M*") is greater than usually seen after active immunization. Taken together, these observations would suggest that equilibration between circulating antibody and the fraction passing into the bronchial tract has not been completed by the end of the second day. Thus this special process appears even slower than the distribution of antibody between the circulating and non-circulating compartments of extracellular fluid, which seems to take about 48 hours.

TABLE 7.

Pathotopic potentiation of passive immunity.
B. Heterologous serum.

Treatment	Infectivity test ΣL	d_x	Log P	Protection P	Potentiation P_X/P_M
R^2	31·54	5·37	2·32	209	1
I^2R^2	25·14	11·77	5·07	118,000	575
Z^2R^2	23·18	13·73	5·97	934,000	3,980
Nil (control)	36·91	$d_{10}=$ 2·32	0·00	1	

Treatments: R=rabbit anti-MEL serum (titre 480), 0·20 ml. peritoneally;
$I=IO_4^-$ M/100 in normal saline, 0·05 ml. nasally;
$Z=ZnSO_4$ 0·08 p.c. in normal saline, 0·05 ml. nasally.
The *index numbers* show the interval, in days, between consecutive treatments.

As regards potentiation of passive immunity, heterologous (rabbit) serum behaves in very much the same way as the homologous serum (Table 7). However, the ratio between basic protection (group "*R*") and enhancement (groups "*I-R*" and "*Z-R*") in this case is significantly higher than with mouse sera; indeed, the values obtained, 575 and 3980, are higher than anything we have seen in the course of our work on active immunity. This would indicate that whatever is causing the discrepancy in the distribution of homologous and heterologous sera, the mechanism is masked and its effect overcome whenever the permeability of the respiratory lining is abnormally increased by the use of pathotopic adjuvants. Similar experiments have been performed also with ferret sera, giving results closely similar to those obtained with rabbit serum. As a general conclusion, then, it may be stated that passive immunity to experimental influenza infection can be successfully enhanced in the mouse by the nasal administration of mild irritants.

DISCUSSION.

The purpose of this study was to test whether passive immunity could be enhanced by the action of pathotopic adjuvants. This question could be answered in the affirmative, and thus the foregoing results may be regarded as the confirmation and extension of our findings relating to active immunity. As the principles underlying pathotopic potentiation as well as the immediate implications of the phenomenon have been considered in detail previously, there is no need here to do more than to refer to the original publications (Fazekas de St.Groth, 1950, 1951; Fazekas de St.Groth, Donnelley and Graham, 1951). It is worth noting, however, that the final tests of the present paper could not be properly designed without extensive preliminary experimentation. These preliminary experiments, all of them connected directly or indirectly with the distribution of foreign antibody in the organism, are of some interest and raise several points calling for discussion.

In principle, there should be no difference between the various routes of passive immunization as one would expect the serum so introduced to spread over the whole of the extracellular space and—given time—attain even distribution. However, since the process of equilibration proved to be remarkably slow, it is not so much the final state but rather the temporal aspects, i.e., kinetics, which gain prominence from the practical point-of-view. A steady state is not reached until four to six days after passive immunization, at a time by which over 90 p.c. of the antibody originally introduced has already been eliminated. The method of intravenous or intraperitoneal immunization thus appears singularly wasteful unless applied in septicaemic or toxaemic conditions where neutralization of the noxious entity can be achieved within the vascular bed. Influenza infection, both epidemic and experimental, does not fall into this class, multiplication of the virus being restricted to the epithelium of the bronchial tract. This fact is strikingly demonstrated also by the enormous (over thousandfold) difference in protective efficacy of passive immunization when applied locally or established by a pararespiratory route (cf. Tables 3 and 4). Consequently, our attention had to be focussed on a special aspect of antibody distribution, namely, that between the lumen of the respiratory tract and the rest of the organism. When determining the latter the titres of circulating plasma were taken, for the sake of convenience, as points of reference. In the case of intraperitoneal immunization such a simplification is justified by the finding that three hours after administration the regression curves of antibody were already indistinguishable from those after intravenous injection. A similar method would not have been admissible with the subcutaneous route, partly because the regression curves coincide only from forty-eight hours onwards, and partly because the distribution appears to be dependent on the volume of the inoculum.

The ratio of antibody in the bronchial lumen and in the blood can be readily determined by titrating the antibody content of bronchial washings and of corresponding sera. However, the actual ratio does not allow direct conclusions regarding the quantity of antibody that permeates the lining of the respiratory tract, as neither side of the dividing membrane is a closed system. The respective rates of antibody elimination may be gauged from some of the experiments. Thus the data of Fig. 3 show that the protective effect of nasally administered antiserum declines at the rate of 1·42 log units per day, this value being uniform over the different dilutions of the serum tested. As on dilution mouse serum shows an average slope of 2·05 log units (Fig. 2 and Tables 3 and 4), it follows that in terms of volume the rate of elimination is $1{\cdot}42/2{\cdot}05=0{\cdot}69$ log units per day, or a half life of 0·43 days. Tests with heterologous sera indicate bronchial half lives of the same order. The rate of elimination of foreign antibody from the blood, on the other hand, is considerably slower and shows also significant differences between sera of different origin. These differences might be due either to a genuine difference in the rate of elimination from the organism,

or to the loss of some of the antibody globulin by attachment to various parts of the vascular bed or the tissues after having left the circulation. Though we can offer no direct experimental evidence to decide the issue, the latter alternative seems the more probable on several grounds. First, the difference between the sera is most striking immediately after immunization, when disappearance of antibody from the blood is most rapid (Table 2 and Fig. 1). This we called the phase of equilibration between blood and tissue spaces, and if elimination of antibody played a dominant role at this stage, one would expect the slope of the curves to stay as steep throughout. In fact, they flatten out from the fifth day onwards. Secondly, it is well known from recent work on isotopically or immunologically labelled proteins that they show widely varying adsorptive affinities to different tissues of the recipient organism. Such a phenomenon could well account for the precipitate drop of fowl antibody as opposed to ferret antibody, when introduced into the blood stream of the mouse. Finally, examination of the slopes of the curves in Fig. 1 shows that in the last phase, that of steady elimination, the difference between the various sera practically disappears, all having a half life between five and six days. This value is in fair agreement with published data on the disappearance of labelled proteins. Being incidental, our observations are not extensive enough to allow a more exact estimate; they point, however, to the complexity of the process and reveal qualitative differences which make it impossible to consider the elimination of antibody—at least in its early stages—a simple monomolecular reaction, as has been done often in earlier studies.

The relative speed of bronchial elimination of antibody, as compared to the drop of serum titres, suggests that although only minute fractions of the total amount are present at any time in the bronchi, yet this route of elimination should not be altogether disregarded. Roughly calculated, antibody lost via the bronchi might amount to 10-20 p.c. of the total in the case of homologous and 2-8 p.c. in the case of heterologous antisera.

This preferential transmission of homologous as opposed to heterologous antibody, evident from their distribution between the circulation and the bronchi, was an entirely unexpected finding. On search of the literature for precedents, two phenomena were found which might have some bearing on the problem. Unfortunately neither of these is strictly comparable with conditions of our experiments, the first of these studies being concerned with the absorption of foreign proteins when introduced by different routes, the second with the penetration of antibody through the extraembryonic membranes of the rabbit foetus. Courtice and his associates (Lake, Simonds and Steinbeck, 1952; Simmonds, 1952; Courtice and Morris, 1952; Courtice, 1952) found definite differences between sera of different provenance, and the effect could be traced to a "permeability increasing factor" present in all fresh sera. This factor was found to be sensitive to heat, being completely destroyed by inactivation (56° C. for 30 minutes) of

the sera, and lost also on prolonged storage of the sera at 4° C. In view of this heat sensitivity it is rather improbable that the discriminative admission of mouse serum into the bronchi would be based on the presence of a "permeability increasing factor". All the sera we used have been inactivated and stored for periods of two to six months before the experiments. Unless mouse serum contained a factor considerably more resistant than that of the other species, this treatment should have uniformly destroyed it in all sera.

The observations of Brambell and his collaborators (1950; 1951a; 1951b; 1952), on the other hand, led to conclusions which seem to fit also our results. Their work, compellingly documented, shows that near the end of the gestation period some of the extraembryonic structures (e.g., the amniotic wall) are hardly permeable to antibodies whether contained in rabbit or heterologous sera. In contrast, the splanchnopleur, while withholding heterologous sera, freely admits rabbit antibodies. This phenomenon is especially clearly demonstrated in their crucial test where, after administration of a mixture of homologous and heterologous serum, the ratio inside the membrane was found to exceed 100 to 1 in favour of the homologous serum. This experiment excludes, of course, the action of a "permeability increasing factor", and the results are best interpreted —as has been done by Brambell *et al.* (1952)—as active transport of the rabbit antibody, while the heterologous globulin passes the membrane only by "seepage" through intercellular spaces. The transport of homologous antibody is not a common property of all types of cells but seems to be restricted to specific tissues. It is tempting to speculate that some of the cells lining the respiratory tract of the mouse might be endowed with similar powers. In these terms the advantage of homologous serum would be accounted for by biological activity, superimposed on the physical laws of partition and diffusion which govern the distribution of heterologous sera. This effect would be noticeable only as long as the natural permeability of the membranes was low; on administration of pathotopic adjuvants the ensuing mild inflammation would allow more antibody to pass through the membrane without comparable increase in cellular activity. By this means the difference between homologous and heterologous sera would be levelled out —a state of affairs which tallies with the findings presented in Tables 6 and 7.

Notwithstanding agreement between assumptions and observation, it should be realized that the above picture is but one of several possible interpretations. Since it has been adopted only after conclusion of the experiments, there was no occasion to test it crucially. Hence it must not be regarded as more than a working hypothesis whose validity is yet to be established. While the preliminary observations thus set an explicit problem in physiology, for the present we have to be satisfied that the differential penetration of antibody is governed by a mechanism both unknown and unexplored.

SUMMARY.

The fate of passively introduced antibody was studied in mice, and regression curves are presented covering the first fortnight after administration of homologous and heterologous sera by four different routes.

In the case of experimental influenza only that portion of antibody is *immunologically active* (i.e., capable of preventing infection) which meets and neutralizes the virus before it could enter a susceptible cell. In mice this represents only 1/300 of homologous antiserum administered parenterally. If heterologous antibody is used the distribution between the circulation and the respiratory tract is even less favourable, approximately 1/2,000. This phenomenon seems to be due to a selective permeability of the lungs for homologous antibody.

By intranasal instillation of certain mild irritants (pathotopic adjuvants) the relative concentration of antibody in the bronchial lumen can be increased by a factor of 5 to 20, with a corresponding rise of resistance to experimental influenza infection. Pathotopic potentiation of passive immunity is equally effective with homologous and heterologous antisera.

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THE HUMAN BLOOD GROUP Tj^a

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Levine (1951) reported the finding of an unusual agglutinin which was termed anti-Tj^a and which disclosed a hitherto unknown human blood group. It was detected in the serum of an elderly female patient who was about to undergo an operation for removal of a carcinoma of the stomach. The serum agglutinated the red cells of all subjects against which it was tested, with the exception of the patient's own and those of her sister. Levine also found that the agglutinin was specifically absorbed by the cells of the tumour tissue, but not by the patient's red cells. To explain the absorption by the tumour cells, the hypothesis was advanced that a mutation had arisen in the tumour with the development of the antigen and this later produced auto-immunization of the patient. A further example of anti-Tj^a was reported from South Africa by Zoutendyk and Levine (1952).

Shortly after publication of Levine's paper, an agglutinin was detected in the serum of a blood donor in Australia and has since been identified as a further example of the anti-Tj^a agglutinin. This paper reports investigations that have been undertaken with the patient's serum, the blood groups of her immediate family, and the results of absorption experiments with tumour cells including those from the carcinoma of Levine's patient. These latter investigations were made possible by generous gifts of serum and dried tumour cells which the authors received from Dr. Philip Levine.

INVESTIGATIONS.

The unusual agglutinin was detected in the serum of a 22-year-old unmarried female blood donor. She was healthy in all respects and her only illness had been untreated whooping cough during infancy. There was no history of pregnancy, blood transfusions or intramuscular injections of blood.

Characteristics of the agglutinin.

The agglutinin was first detected by the indirect Coombs test against Rh positive cells, but it also reacted when tested against cells suspended in saline or albumin. When first obtained it was found to haemolyse red cells, a property which was destroyed by inactivation of complement at 56° C. for 20 minutes and which was restored by the addition of fresh group AB serum. The serum agglutinated in every instance the red cells of 1,068 group O white Australians, but it did not react with the subject's own cells. It had a titre varying between

16 and 32 when the test cells were suspended in saline, and approximately the same titre when the cells were suspended in 30 p.c. bovine albumin. Indirect Coombs tests were strongly positive. The agglutinin was active at 37° C., 20° C. and 4° C. Saliva and serum from positive reactors, including patients with carcinomata, failed to neutralize the agglutinin, indicating the absence from body secretions of soluble polysaccharides with the specificity of the red cell agglutinogen.

It has not been possible to absorb anti-A and anti-B from the subject's serum. To demonstrate that the agglutinin does act on the other groups, it was eluted at 56° C. for 5 minutes from washed agglutinated group O cells. The eluate then agglutinated cells of all groups. For subsequent work a solution of A and B blood group polysaccharides was mixed with the serum to neutralize the anti-A and anti-B agglutinins.

The subject's blood group was found to be group O, cdE/cde MSNs, Kell negative, Le(a) positive, Lu(a) negative and Fy(a) negative.

Racial distribution.

The neutralized serum has agglutinated in every instance the red cells of 1,208 white Australians, 412 New Guinea natives, 96 Fijian natives, 100 New Caledonians, 33 Gilbertese, 198 natives of the British Solomon Islands, 337 natives of South Korea, 422 Filipinos, and 94 Australian aborigines.

Homogeneity of antibody.

The possibility that the universal reactivity of the serum was due to the presence of a number of distinct antibodies was investigated by absorbing the serum with varying amounts of red cells obtained from subjects of different blood group combinations. No evidence of heterogeneity of the antibody was obtained, and furthermore, eluates obtained from the cells used for absorption caused agglutination of all cells. No differences in titre were obtained when the serum was titrated against a panel of red cells suspended in physiological saline and in 30 p.c. bovine albumin.

The agglutinin did not show the specificity of any of the known or predicted blood group agglutinins. It reacted to the same degree against red cells coated with the incomplete antibody of acquired haemolytic anaemia as it did against red cells selected at random, suggesting that it is not related to this latter antibody. The red cells of the propositus showed strong agglutination with the species agglutinins of equine, bovine and ovine sera. Finally red cells agglutinated by pertussis haemagglutinin and others coated with pneumococcus Type III polysaccharide absorbed usual amounts of the atypical agglutinin.

Family investigations.

The members of the subject's immediate family were examined for blood groups with the results shown in Fig. 1. All were tested with the serum of the

propositus and all reacted with the exception of her 19-year-old sister. The serum of this subject—the third sibling—was also found to contain an antibody having properties identical with those of the original antibody. The sera of the other members of the family were examined but no atypical antibodies were detected. The red cells of the parents and siblings were also titrated against the serum of the propositus but no dosage effects were observed. The 19-year-old sister was healthy and had not suffered in the past from any illness requiring medical attention.

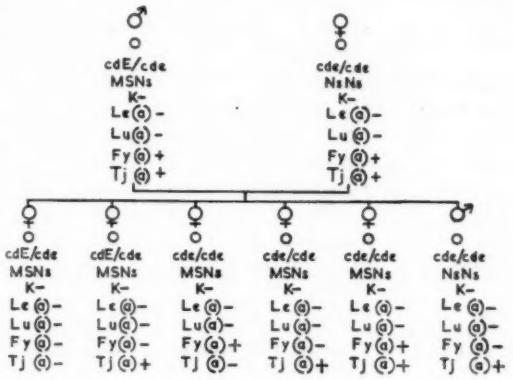

Fig. 1.

Comparison with anti-Tj^a.

A sample of serum from Levine's first patient (Levine, 1951) was active on arrival from U.S.A., but failed to react with the red cells of the two sisters. Furthermore, Zoutendyk and Levine (1952) reported that the serum from the 22-year-old Australian subject was identical with the anti-Tj^a serum when tested in U.S.A.

Relationship to carcinomatous tumours.

In this work the technique employed by Levine (1952) was followed. 10 mg. of the dried tumour tissue were mixed with 1 ml. of serum in a 0·8 cm.×7·5 cm. test tube and shaken on a mechanical shaker for 3 hours at room temperature. The shaking machine was of the type described by Dacie (1950) for mixing dilutions of blood prior to counting the erythrocytes. The tubes were then placed in the cold room overnight, and shaken for a further 60 minutes at room temperature next morning. They were finally centrifuged to remove the tumour tissue before titration. In some instances the quantities were reduced to 5 mg. of dried tissue and 0·5 ml. of serum. The unabsorbed sera acting as controls (two anti-D, one anti-E and one anti-A) were also treated as were the absorbed sera. The results of absorptions with the tumour tissue supplied by Levine are shown in Table 1. It is clear that the tissue absorbs both the anti-Tj^a and the Australian sera, and appreciable amounts of the anti-E and of one anti-D serum. The strength of the reactions with the second anti-D serum and the anti-A serum was reduced after absorption, but the end points of the titrations were not significantly different.

TABLE 1.

Absorption of sera with tumour tissue.

Serum	Absorption	Titration in saline 1	2	4	8	16	32	64	128	256
Anti-Tj^a	Not absorbed	++^{v}	++^{v}	++	+	—	—			
Diluted 1 in 2·5	Absorbed with tumour cells	++^{v}	w	—	—	—	—			
Australian serum	Not absorbed	++^{v}	++	w	—	—	—			
Diluted 1 in 2	Absorbed with tumour cells	++	+	—	—	—	—			
Anti-D	Not absorbed	++^{v}	++	w	—	—	—			
Diluted 1 in 2	Absorbed with tumour cells	—	—	—	—	—	—			
Anti-D	Not absorbed	++^{v}	++^{v}	++^{v}	++^{v}	++^{v}	++^{v}	++^{v}	(+)	—
Undiluted	Absorbed with tumour cells	++^{v}	++^{v}	++^{v}	++^{v}	++^{v}	++	+	—	—
Anti-E	Not absorbed	++^{v}	++^{v}	++^{v}	++^{v}	++	w			
Diluted 1 in 2	Absorbed with tumour cells	++	++	w	?	—	—			
Anti-A	Not absorbed A_1 cells	++^{v}	++^{v}	++^{v}	++^{v}	++^{v}	++	+	w	—
	Not absorbed A_2 cells	++^{v}	++^{v}	++^{v}	++^{v}	+	(+)	+	+	—
Diluted 1 in 6	Absorbed with tumour cells A_1 cells	++^{v}	++^{v}	++^{v}	++^{v}	++^{v}	++^{v}	++	+	?
	A_2 cells	++^{v}	++	++	++	+	+	w	w	—

Three carcinomatous tumours and a portion of normal gastric mucosa, obtained either at operation or at a post-mortem examination, were also used after lyophilization to absorb both the anti-Tj^a and the Australian sera. In all instances absorption was as marked as with the American tumour cells and some non-specific absorption of anti-E was also shown by one tumour.

DISCUSSION.

The agglutinin found in the sera of two healthy young females appears to be identical with the anti-Tj^a agglutinin described by Levine (1951). It has one of the characteristics of an immune agglutinin, in being associated with a haemolysin, but no history of parenteral antigenic administration was obtained. Unlike Levine's patient, neither subject investigated was found to have localized mutant tissue which could cause auto-immunization.

The anti-Tj^a agglutinin reacts with the red cells of all but five subjects amongst several thousands of different ethnic origin that have been tested in America (Levine, 1951), in South Africa (Zoutendyk and Levine, 1952), and in Australia. This fact could be explained on a simple genetical basis by assuming that the agglutinogen Tj^a is of a very frequent occurrence. The heterozygous

Tj^a Tj^b and the negatively reacting Tj^b Tj^b individuals would be extremely rare, and to date Tj^b Tj^b individuals have been detected only amongst those of European descent. However, the fact that the five negative reactors were found in three different continents raises the possibility that mutations have occurred in these individuals with deletion from a chromosome of an otherwise universally present gene which produces the red cell agglutinogen Tj^a.

The present investigation has confirmed Levine's finding that the tumour cells from his patient absorb anti-Tj^a from both the American and the Australian sera. It has also shown that these tumour cells absorb other immune agglutinins including anti-E. The red cells of the patient from whom the tumour was obtained did not possess Tj^a or E antigen, and it must be supposed either that the absorption is non-specific in nature or that two antigens, Tj^a and E, have arisen by mutation in the tumour tissue, each capable of absorbing specifically the corresponding anti-serum. If this latter hypothesis is entertained it is difficult to understand how auto-immunization occurred from one and not from the other mutant antigen in Levine's patient. Other carcinomatous tumours used in the present work avidly absorbed anti-Tj^a, as well as other immune agglutinins. In some instances other agglutinins were absorbed although the corresponding antigens were not present in the red cells of the patient from whom the tumours were obtained.

SUMMARY.

An unusual agglutinin was detected in the sera of two siblings. It reacted with the red cells of all individuals with the exception of those of the propositus and her sister, and is apparently identical with the anti-Tj^a agglutinin. Tumour cells from Levine's patient absorbed the Tj^a agglutinin from the sera of both Australian subjects as well as other immune agglutinins from test sera. The patient did not possess the E antigen in her red cells, but anti-E was absorbed by the tumour cells. The significance of these findings has been discussed.

Acknowledgments. The authors are greatly indebted to Dr. Philip Levine for identifying the agglutinin as anti-Tj^a and for permitting the subsequent investigations by his generous gifts of tumour cells and serum.

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THE PANCREATIC LESION IN ADULT MICE INFECTED WITH A STRAIN OF PLEURODYNIA VIRUS

1. ELECTRON MICROSCOPICAL OBSERVATIONS

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In a previous communication from this laboratory (Dineen and Barter, 1953) a series of changes, including the presence of curious laminated cytoplasmic bodies, were described as occurring in the pancreatic acinar cells of adult mice infected with a local strain of Pleurodynia virus (designated as the PL5 strain by Atkinson, Dineen and Robertson, 1951). Because these changes seemed interesting, complex and somewhat unusual, and as it was not possible to make out many fine structural details with the visible-light microscope, a study was undertaken with the electron microscope in thin sections of the infected pancreas.

METHODS.

Preparation of virus suspensions. For inoculation of adult mice, a 20 p.c. muscle suspension was prepared in chilled physiological saline from the limbs of infant mice which were moribund from the 19th passage of the PL5 virus. The crude suspension was spun in the "Spinco" ultracentrifuge at 15,000 R.P.M. for 10 minutes, and the supernatant harvested. After the demonstration of bacteriological sterility by culture, this preparation was inoculated intraperitoneally into adult mice in doses of 0·2 ml. A control 20 p.c. suspension was prepared in an identical way from the muscles of non-infected infant mice.

Histological procedures. The methods used in this laboratory for preparing tissues for examination in the electron microscope have been described in a previous paper (Robertson, 1954). In addition, portions of each pancreas were taken into 10 p.c. formalin and after embedding in paraffin were stained with haematoxylin-eosin and haematoxylin-phloxine-tartrazine for examination in the visible-light microscope.

Animals. 62 male albino mice, aged 6 to 8 weeks, from a stock colony were used. There were 40 infected animals, 6 control animals injected with a non-infected 20 p.c. muscle suspension, and 10 normal controls. In addition, 6 animals were used in a small preliminary experiment designed to exclude the possibility that the cellular changes seen were merely the result of the death of the cell with ensuing post-mortem degeneration. Animals were killed at intervals beginning at 6 hours and ending at 12 days after inoculation. Two blocks from the pancreas of each animal, fixed in buffered 1 p.c. osmic acid solution, were cut and examined in the electron microscope.

RESULTS.

Naked-eye changes. 18 hours after intraperitoneal inoculation of the PL5 virus the adult mouse shows no alterations in appearance and behaviour, and on sacrifice the pancreas appears quite normal to the naked eye. At 24 hours,

although the animal still appears well and behaves normally, the pancreas shows definite oedema, with slight wasting and pallor of the lobules, which have taken on a very characteristic ivory colour instead of the pale yellow-brown of the normal organ. These changes are more marked at 28 hours, at which time the pancreas is definitely shrunken, and wasting proceeds steadily thereafter until the death of the animal (which by then has lost a considerable fraction of its initial body weight (Dineen and Barter, 1953) at 12 days or later. Only occasional animals survive indefinitely. No changes occur in animals injected with the control (non-infected) muscle suspension, or in the normal controls.

Microscopic changes. Again no pathological changes can be seen in control animals. In infected animals the morphological reaction in the pancreas has three main phases. There is first a mainly degenerative phase, in which the great majority of the acinar cells undergo complex internal changes and then disintegrate. This phase occupies approximately the first 48 hours, and is followed by a second phase of inflammatory reaction which is almost exclusively mononuclear in character, and which after about the fifth day passes into a third and final phase of residual fibrosis. A good deal of temporal overlap occurs between the three phases.

The degenerative phase.

At 12 hours after inoculation no definite lesions can be seen in sections of the pancreas, but by 18 hours diffuse or focal alterations are beginning in the cytoplasm of the acinar cells. Only a proportion of cells is affected in the early stages, but the number of normal cells visible steadily and rapidly decreases with time. Not infrequently a single acinar cell shows focal superimposed on diffuse cytoplasmic abnormality.

The focal cytoplasmic lesion begins with an increase in vacuolation of a region of the cytoplasm. The cytoplasmic double membranes, which are normally very conspicuous in the pancreatic acinar cell (Dalton, 1951; Sjöstrand, 1953; Robertson, 1954) separate to form a system of elongated vacuoles which appear to enlarge by breaking into pre-existing vacuoles. A focal lesion of this type is illustrated in Fig. 1, from an animal which had been infected for 24 hours.

No particles comparable in size to virus particles have been seen in early lesions of this type, although it is of course entirely possible that if only a few particles are present they may not lie in the plane of the section.

With further enlargement of the focal lesion a body made up of a series of concentric laminae is often formed. These bodies, which will be hereafter termed laminated bodies, correspond to the bodies described by Dineen and Barter (1953). Some of them are present at 18 hours in a small proportion of acinar cells, but at 24 hours they are much more numerous, and they reach their greatest number, size and complexity of structure at 36 to 48 hours. They are

composed of several, often many, laminae, each of which is made up of a thin layer of cytoplasm between two denser membranes. Very complex appearances result, as can be seen from Figs. 2 to 11, and all gradations exist between the focal cytoplasmic change resulting in the formation of a laminated body, and the more diffuse cytoplasmic vacuolation shortly to be described. In a few instances small dense particles of dimensions (approximately 50-75mμ) such that they might be virus particles, were seen in the centres of complex laminated bodies. A good example is depicted in Fig. 5, from an animal infected for 20 hours. In the enlargement (inset) of the central part of this lesion it may be seen that some of the larger particles present are "hollow" spheres.

The central region of a laminated body may have a variable structure. It may appear essentially empty (Fig. 8). It may consist of a segregated portion of diffusely vacuolated cytoplasm (Figs. 3, 4 and 13). It may contain a granule, perhaps a zymogen granule or altered mitochondrion (Fig. 7). However, no support was found for the hypothesis that a laminated body arises round a cell organelle as nucleus. In many cases mitochondria can be seen (Fig. 5) apparently fortuitously caught up in or pushed aside by laminated bodies and networks without showing any definite alterations in the characteristic mitochondrial morphology. The same applies to zymogen granules (Figs. 6 and 7). Again, however, the limitations of this histological method must be borne in mind, and without a reliable method which will make available a large number of serial sections for study in the electron microscope, no definite statement can be made on the nature of the core or nucleus of the laminated body—if one exists. The evidence of Fig. 5 suggests that at least some virus particles may be present.

The second main type of cytoplasmic change is a diffuse vacuolation which appears to represent an exaggeration of the normal vacuolation which may sometimes be seen in an acinar cell. As the vacuoles enlarge their lumina become very clear, offering almost no obstruction to the electron beam, while their bounding membranes become applied to those of their neighbours. In consequence, the cell cytoplasm, becoming filled with these cavities, takes on an appearance which will be termed a network (Figs. 2 to 5, 7 and 8). Although the material within the vacuoles is of very low electron density, the vacuolated cytoplasm of cells showing this change appears deeply basophilic when stained with haematoxylin and eosin in paraffin sections and examined in the visible-light microscope.

Many acinar cells show combinations of network and one or several laminated bodies. Fully-developed laminated bodies have not been seen in cells which have an otherwise normal-appearing cytoplasm. Invariably a diffuse vacuolation surrounds the body, although in a few instances (see Fig. 6) this is not of marked degree. However, in many cases the network appearance can be seen without any focal lamination. The possibility of the occurrence of focal lamination outside the plane of section in cells of this latter type is of course not excluded by this observation.

A third type of cytoplasmic reaction, which was seen infrequently and which appeared different from the formation of networks and laminated bodies, although it was at times associated with both these latter reactions in the same cell, was the occurrence of dense inclusions (Figs. 12 and 13). These are large rounded bodies, up to 5μ in diameter, usually lying near the nucleus. The dense inclusion shown in Fig. 12 indents the nucleus of a cell in which the remaining cytoplasm appears essentially normal apart from a doubtful increase in vacuolation. Its outline is fairly regular, and some details of its internal structure can be made out. It contains several small clear circular "vacuoles" which vary in size approximately from 100 to 200 mμ, as well as some more irregular clear internal gaps and some much larger peripheral vacuoles. Small irregular areas of increased electron density are also apparent.

The similar body shown in Fig. 13 is smaller, and contains more, and more regular, internal vacuoles. Furthermore, a large round oval granule is visible near its centre, which possibly represents a swollen mitochondrion. The cytoplasm of the cell containing this dense inclusion also shows a diffuse vacuolation as well as a laminated body segregating a mass of vacuolated cytoplasm. All three types of cytoplasmic reaction are thus present in this single cell.

The ultimate fate of the dense inclusions has not been determined.

With the passage of time the laminated bodies come to show still more complex appearances. At 36 hours, and still more at 48 hours, swellings, either terminal (Figs. 7, 8 and 9) or central (Fig. 8) or both, may appear in the laminae of the body. (It seems possible that laminae with free ends are produced by rupture of the thin layer of cytoplasm intervening between two vacuoles.) Not all laminated bodies show this change, but most of them do so to a slight degree, and a rather small proportion to a marked degree. Swollen fragments of the membranes, usually appearing to arise from the free ends of laminae, then seem to break off into the intermembranous spaces in the form of ovoid or somewhat irregular and elongated bodies. Figs. 7, 9, 10 and 11 illustrate the complex appearances which may result. In Fig. 9 dense bulbous swellings of the free ends of laminae can be seen at D3 and D8, and many dense irregular rounded or elongated bodies lie apparently free within the intermembranous spaces. To the right of Fig. 9 a large macrophage may be seen closely applied to the laminated body. Portion of a macrophage can also be seen to the upper right in both Figs. 7 and 10. Very frequently in fact, when a laminated body is seen undergoing this complex type of disintegration, one or more macrophages is present in contact with it.

However the small loose bodies shed from the laminae are not always of dense homogeneous structure. They are sometimes, as is shown in Fig. 10, comma-shaped, hollow spheres, signet-ring forms, or may be even larger masses of cytoplasm (Fig. 11).

Ultimately, with increasing oedema together with disintegration of laminae, only a few fragments of the originally complex laminated body remain.

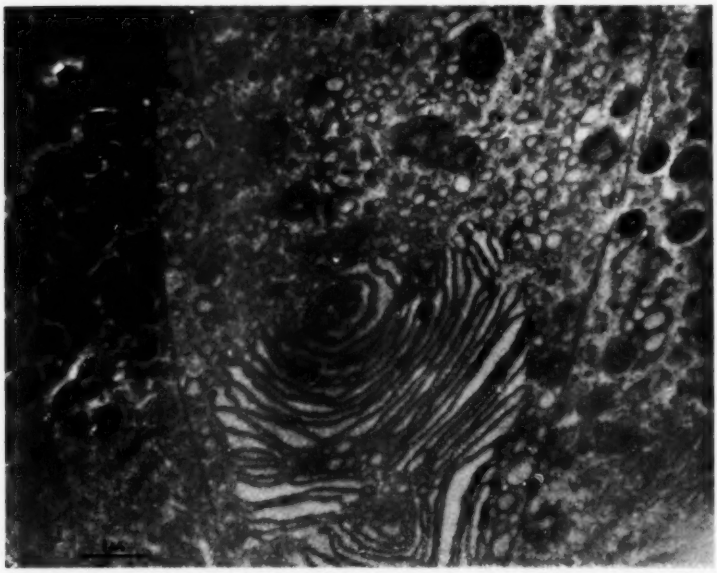

Fig. 1. Early focal lesions (20 hours) in an acinar cell. Several mitochondria of normal appearance are visible in the upper part of the affected cell.

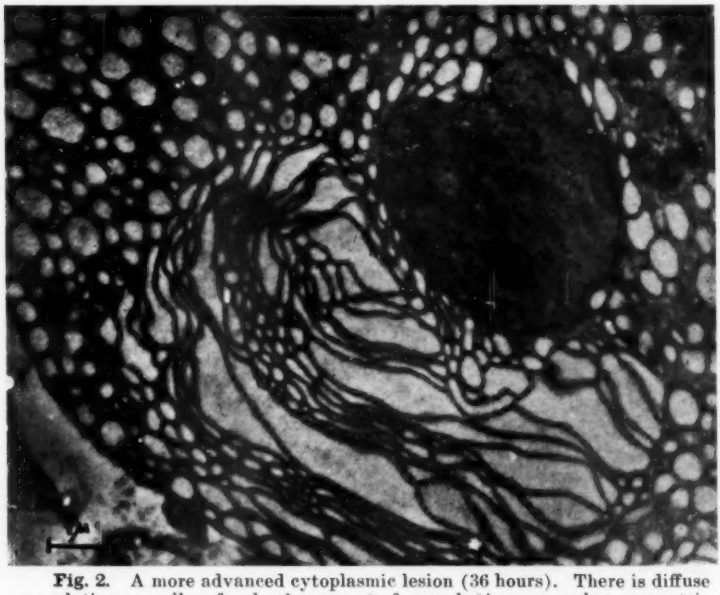

Fig. 2. A more advanced cytoplasmic lesion (36 hours). There is diffuse vacuolation as well as focal enlargement of vacuoles in a somewhat concentric fashion. Pyknotic changes are present in the nucleus. To the right of the nucleus there is a swollen mitochondrion (?), and in the extreme upper right corner is a clump of small particles.

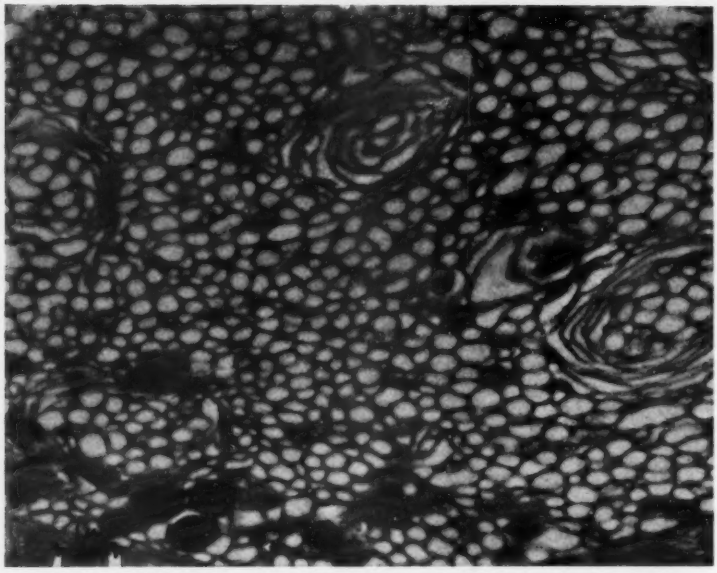

Fig. 3. Affected cytoplasm at 48 hours. Focal lamination is present on a background of diffuse vacuolation (network).

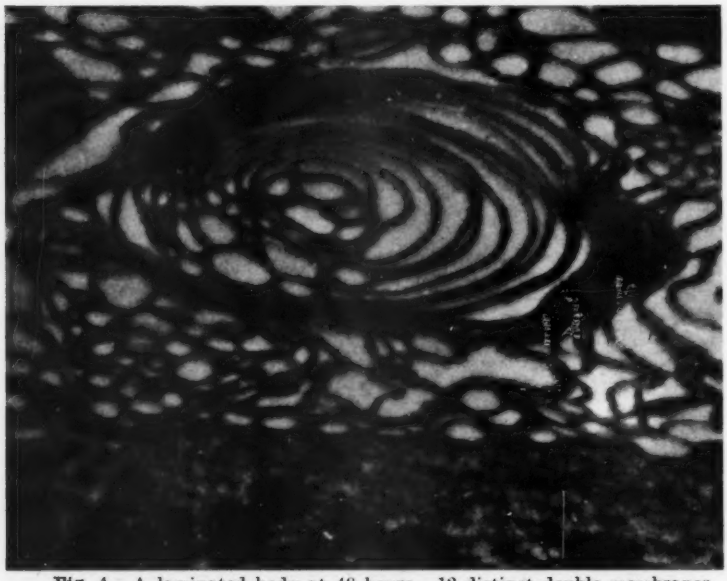

Fig. 4. A laminated body at 48 hours. 12 distinct double membranes are present in the pole of the body at the right. Diffuse vacuolation surrounds the body. Portion of an apparently unaffected cell is present in the lower part of the figure.

Fig. 5. A complex laminated body and network. Small dense particles are visible at J6, which on enlargement (inset) are seen in some instances to have light centres. 2 pyknotic nuclear masses are visible, as well as several mitochondria (A4, C8, C6, H4, J4). The appearances of the latter are within normal limits.

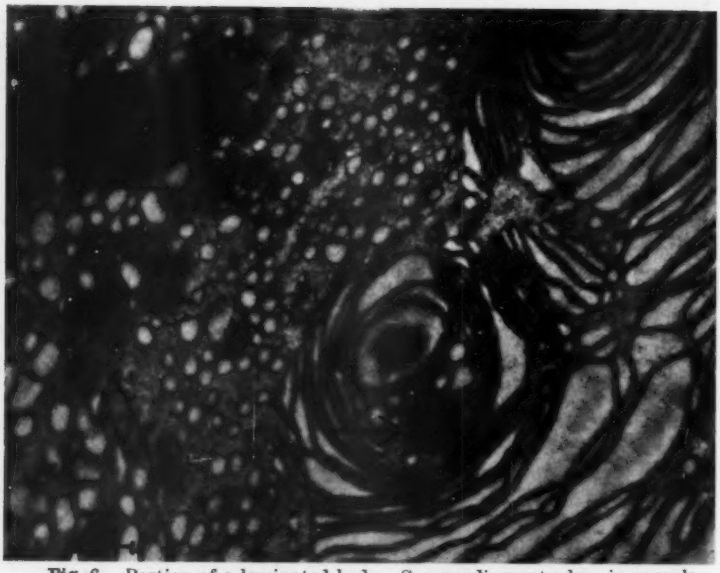

Fig. 6. Portion of a laminated body. Surrounding cytoplasmic vacuolation is not marked. The arrow indicates a zymogen granule of normal appearance.

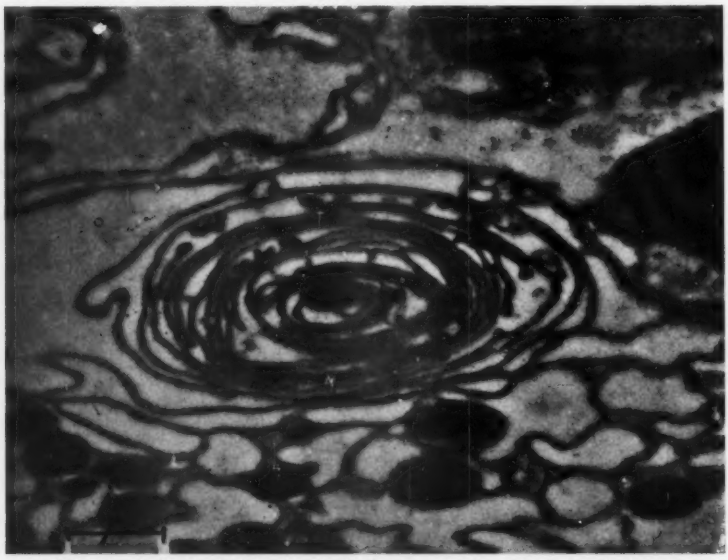

Fig. 7. A laminated body at 48 hours. In the centre is what appears to be a zymogen granule. Many of the concentric double laminae have small bulbous swellings on their free ends, a few of which have apparently been shed into the interlaminar spaces. Portion of the densely pyknotic nucleus is visible at the extreme right centre, several zymogen granules in diffusely vacuolated cytoplasm in the lower parts of the figure; and portion of a macrophage in the upper right corner.

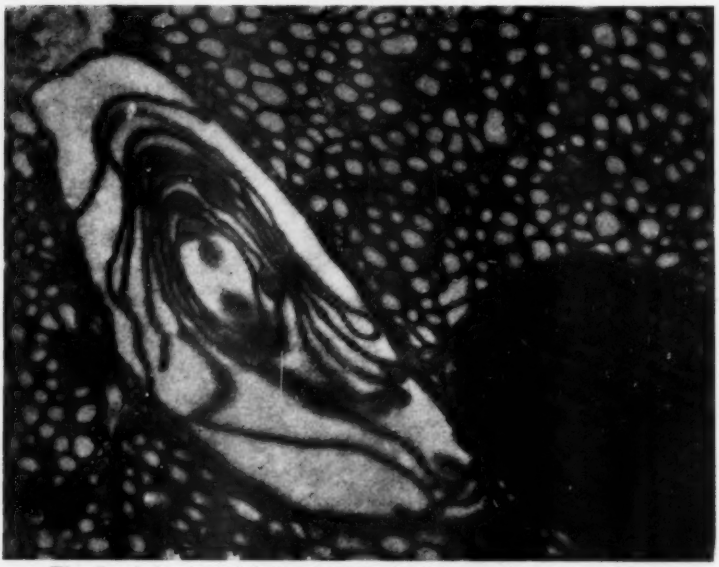

Fig. 8. A laminated body (36 hours) with prominent central and terminal swellings on the laminae. The pyknotic nucleus at the lower right.

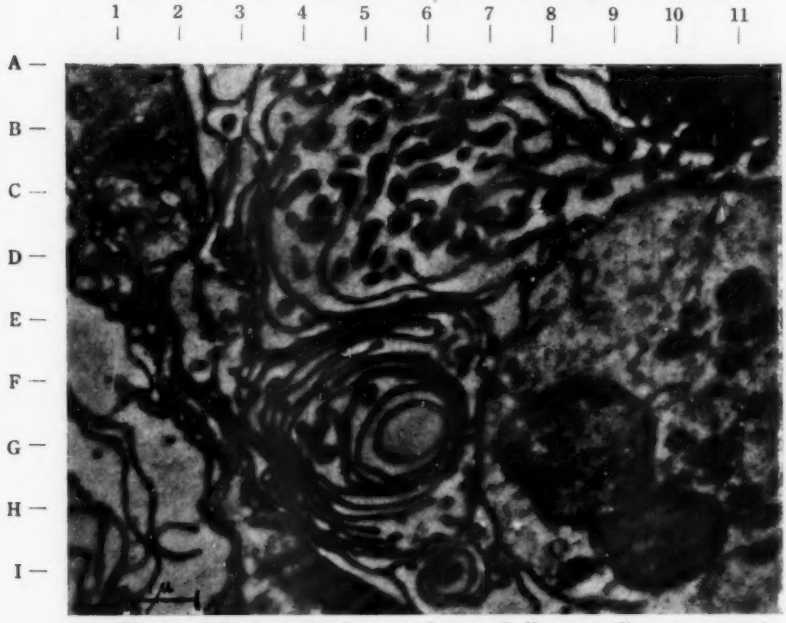

Fig. 9. A laminated body at 48 hours. Bulbous swellings are present on the ends of laminae at D3 and D8. The pyknotic nucleus is at B10. Many large dense irregular apparently free bodies are present in the area centred at C6. At the lower right a macrophage can be seen applied to the degenerating laminated body.

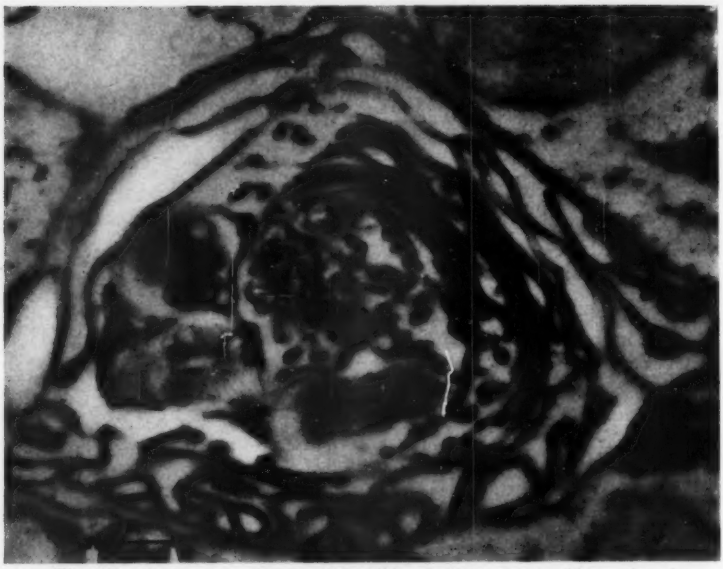

Fig. 10. A degenerating laminated body (62 hours). Many free irregular fragments of variable density are present. The laminae themselves vary greatly in density. Portion of a macrophage can be seen at the upper right.

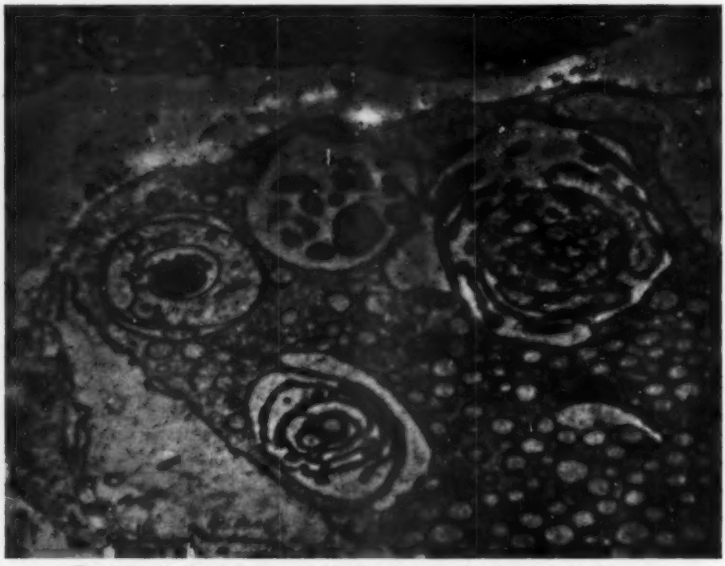

Fig. 11. 4 focal lesions of rather unusual type, at 62 hours.

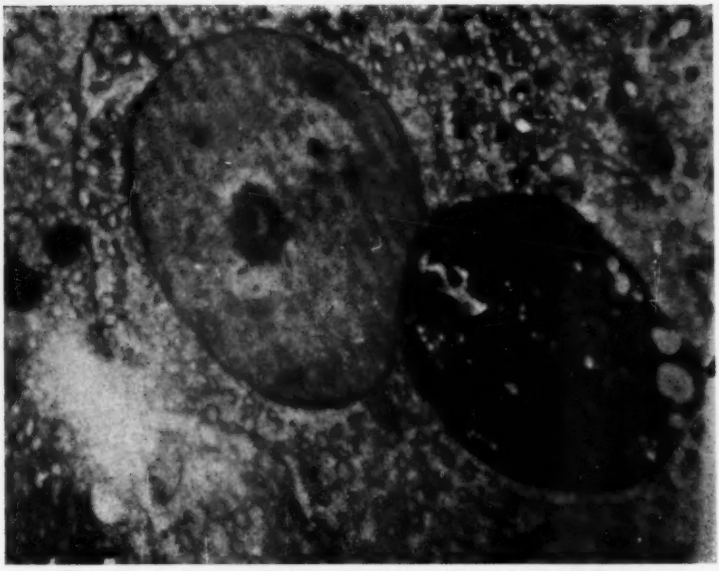

Fig. 12. A dense inclusion adjacent to the nucleus (48 hours).

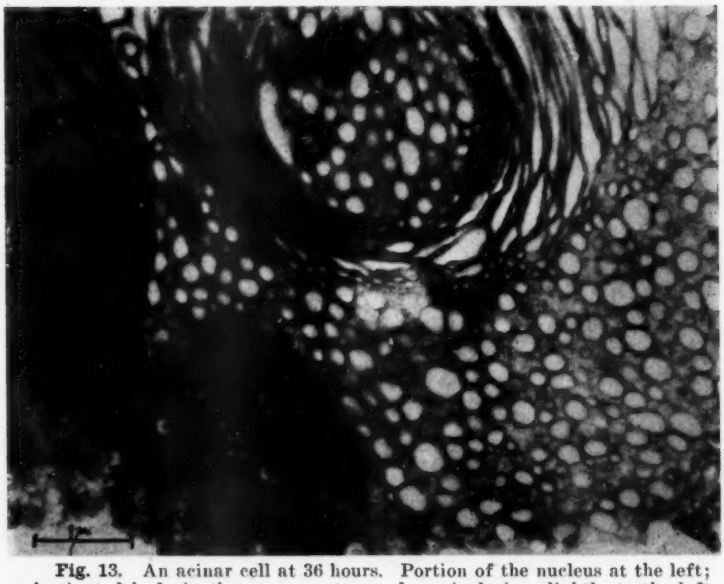

Fig. 13. An acinar cell at 36 hours. Portion of the nucleus at the left; a laminated body in the upper centre; a dense inclusion slightly to the left of the lower centre, containing many small vacuoles as well as a larger regular body surrounded by a thin dense membrane.

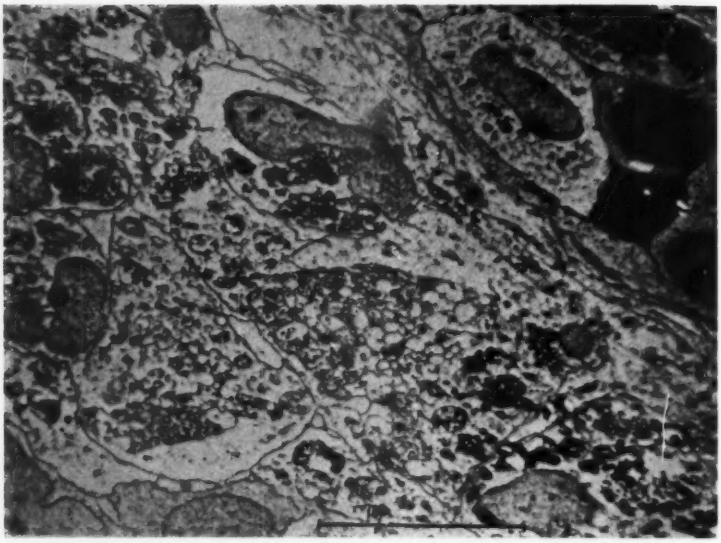

Fig. 14. Macrophagal replacement of acinar cells at 82 hours. Much intracellular debris.

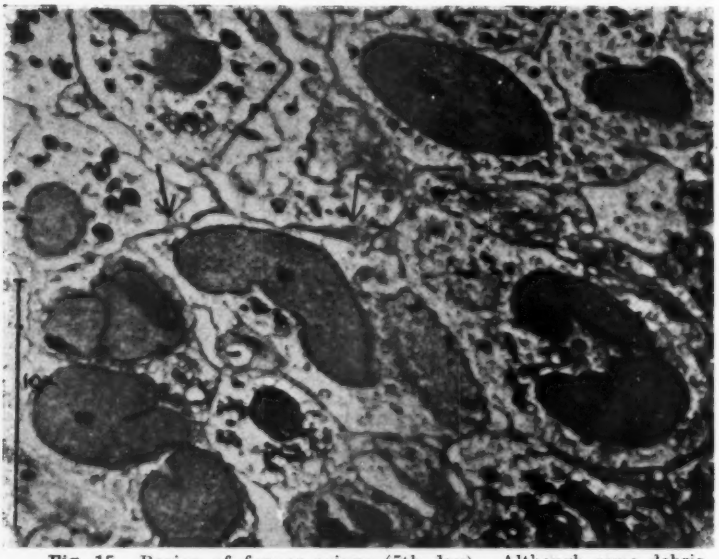

Fig. 15. Region of former acinus (5th day). Although some debris can still be seen in the cytoplasm of the macrophages, their nuclei are becoming more elongated and an intercellular matrix is beginning to appear (arrows).

Changes in the nuclei of the acinar cells occur *pari passu* with those in the cytoplasm. The first change, seen at about 18 hours, is a decrease in the size of the nucleoli, followed soon afterwards by a wrinkling of the nuclear membrane and a gradual general increase in density of the nucleoplasm (Figs. 5, 8 and 9), until by 36 to 48 hours, when cytoplasmic changes are most intense, the nucleus is quite definitely shrunken, irregular and pyknotic. Soon afterwards it breaks up into dense fragments which are ingested by the mononuclear phagocytes which invade the affected acini in large numbers.

Changes in the mitochondria and zymogen granules of affected cells are not striking, and have already been described in part. Fig. 7 illustrates what appears to be a zymogen granule in the centre of a laminated body, and Fig. 5 shows mitochondria involved apparently fortuitously in networks and laminations. Fig. 13 shows what is interpreted as a swollen mitochondrion in the centre of a dense cytoplasmic inclusion. Islets of Langerhans and duct cells are not apparently affected, and in the later stages may be seen intact in the midst of completely necrotic acinar tissue.

The phase of inflammatory reaction.

The inflammatory reaction is almost exclusively mononuclear, and begins quite early, at about the 12th hour, with the presence of a few rounded macrophages in the fibrous septa of the organ. Granular leucocytes, although they do occur, are seen very infrequently among the degenerating acinar cells at any stage of the reaction, although they are often present in small numbers in the septa of the gland. By the 24th hour some macrophages have begun to penetrate the acini, and at 48 hours are very numerous among the degenerating acinar cells, applying themselves to laminated bodies and ingesting the fragments of pyknotic nuclei. As the acinar cells collapse the macrophages take their places in the reticular framework of the organ, and often come to be very large, and filled with a great number of fragments.

The fibrous reaction.

This last phase of the pancreatic lesion occurs quite quickly, and is well established after the 4th day. Just before this time the organ is quite cellular and is made up of a dense aggregation of tightly-packed macrophages (Fig. 14), many of which still contain fragments of degenerated acinar cells. The mononuclear cells then appear to change their characters. Their nuclei become more ovoid, and the nucleoli a little more prominent. At the same time an intercellular matrix appears (Fig. 15). This matrix has not been certainly identified, but it contains fine hair-like fibres, so that it is probably reticulum (collagen). The whole appearance suggests, though it does not prove, the transformation of macrophages *in situ* into fibroblasts. Mitotic divisions are seen only very rarely among the cells that penetrate the necrotic acini, and it does not appear likely

that the very rapid appearance of large sheets of fibroblasts can be accounted for entirely by the streaming in of these cells from the perivascular and interacinar connective tissue, though this latter process does indeed occur.

By the seventh day the organ is a pale shrunken fibrous remnant in which only islets of Langerhans and empty ducts are conspicuous.

DISCUSSION.

The much more detailed morphological analysis made possible by the electron microscope has amplified the description of the pancreatic lesion by Dineen and Barter (1953) without altering it in principle, and has emphasized that the reaction is one of great complexity, about which at present only the most tentative conclusions can be drawn. The first step towards understanding the reaction is obviously to attempt to relate the morphological changes to the stages of multiplication of the virus. Accordingly the amount of virus extractable from the pancreas at various times during the development of the lesion has been determined by titration in litters of infant mice (Dineen, personal communication). At 6 hours after intraperitoneal inoculation the LD_{50} for infant mice of a pancreatic extract made by grinding the pooled pancreases of 5 adult animals with alundum was $10^{-3.9}$ gm. (wet weight), rising rapidly to a peak between the 31st and 48th hour. (The LD_{50} at 31 hours was $10^{-7.7}$ gm., and at 48 hours $10^{-6.6}$ gm.) The time of peak virus content is also that at which laminated bodies and networks reach their greatest numbers and complexity. At 4½ days there has been a hundred-fold fall in virus content from the peak value to an LD_{50} of $10^{-5.3}$ gm., at which time the acinar cells are almost completely necrotic and replaced by mononuclear cells. By the 7th day, when fibrous replacement is virtually complete, the LD_{50} has fallen to $10^{-2.7}$ gm. However, even at the 31-hour stage when the virus titre is at its highest, particles of such dimensions that they might be virus particles are visible very rarely. It may be, however, that any such particles occur in clumps (as in Figs. 2 and 5) which would not be seen unless the plane of the section passed through the clump.

Electron microscopical studies similar to this present investigation have been made by Gaylord and Melnick (1953) on the morphological changes induced by pox viruses. They, too, found complex appearances which were hard to interpret, but laminated bodies and cytoplasmic networks did not occur in their material, perhaps because the cells they studied do not normally possess very many cytoplasmic double membranes, whereas the pancreatic acinar cells which were the subject of the present study contain very many of these membranes, which might reasonably be expected to show changes during the reaction to a cytoplasmic virus such as the PL5 virus.

It seems clear that the main morphological reaction in the acinar cell to PL5 infection is cytoplasmic, and involves in most cells an exaggeration of vacuolation to form laminae which may take on a more or less concentric form. It is tempt-

ing to regard the latter reaction (the laminated body) as one which in some way circumscribes the virus, and Fig. 5 affords some slight suggestion that this may indeed be the case. It is further considered, from the evidence afforded by appearances such as that of Fig. 4, that a laminated body is formed not only by a rearrangement and separation of pre-existing cytoplasmic double membranes but also by elongation and extension of the membranes by the addition of more membranous material. One pole of the body shown in Fig. 4 (on the right in the figure) is made up of 12 distinct closely apposed double layers. It seems unlikely that such an appearance could result from mere rearrangement of existing membranes.

The nature of the small irregular fragments formed by disintegration of the laminated body is quite obscure. They are nearly all much too large to be virus particles. The comma-shaped or more irregular fragments may represent nothing more than the manner in which a membranous structure disintegrates. However, the more solid bodies, particularly those formed terminally on the laminae, convey the impression of the actual addition of material to the structure, though they could be produced by the contraction of the lamina after its rupture.

However, many grossly abnormal cells show only the diffuse cytoplasmic vacuolation termed a network, perhaps with one or several foci of superimposed lamination, but with no visible particles. The evidence at present available gives little information on the intracellular distribution of the virus at any stage. Even when the virus content of the organ is greatest it is possible that only a relatively few particles are present in any one cell, perhaps clumped, and lying outside the plane of the section.

The dense inclusions which were occasionally seen resemble those illustrated by Gaylord and Melnick (1953) in a proportion of corneal cells infected with vaccinia. These authors were able to show what they considered to represent the digestion away of the dense matrix of the inclusion to reveal virus particles which had developed within. In the present material dense inclusions were seen quite infrequently, and it was not possible to come to any conclusions on their subsequent fate.

The nuclear changes in the acinar cells appear to be secondary to the cytoplasmic changes. Decrease in the size of nucleoli, with wrinkling of the nuclear membrane and a general increase in the electron density of the nucleoplasm occur relatively early, and are well established when the cytoplasmic changes are at a maximum. Thereafter the nucleus rapidly becomes increasingly pyknotic, and breaks into fragments of which the remnants are ingested by mononuclears.

As previously noted (Atkinson, Dineen and Robertson, 1951; Dineen and Barter, 1953) the pancreatitis induced by this virus spares the islets of Langerhans. This is especially evident in the electron micrographs, when intact islets of normal appearance are often seen lying amid completely necrotic acini. In a few animals, however, the islets eventually seem to be strangled in the fibrous

tissue, and a considerable terminal rise in blood sugar has been occasionally noted (Dineen, personal communication). However, the inanition induced by the loss of the external secretion of the pancreas seems to play a large part in the usually fatal outcome of the infection.

The inflammatory and fibrous reactions which succeed the degenerative phase are in each instance both striking and rapid. Particularly striking is the apparent affinity of the laminated bodies for macrophages (Figs. 7, 9 and 10). By the fourth day, although the pancreas is by then considerably shrunken, a paraffin section examined in the visible-light microscope shows that the organ is quite cellular, and the electron microscope shows further in thin sections from this stage that most of the cells are macrophages, filled with debris from necrotic acinar cells. At this stage, though there has been a definite decrease in the virus content of the organ, a not inconsiderable amount of virus is still present.

Quite rapidly thereafter, and without more than an occasional mitotic division being visible in sections, fibroblasts take the place of the macrophages and fibres appear between them. It has long been held by some workers, mainly on the basis of tissue culture experiments (Maximow, 1927, 1928; Bloom, 1928; Fischer, 1946), but also by many histopathologists, that a transformation of mononuclears into fibroblasts might occur. However others, also working with tissue cultures, have either opposed the transformation (Hall and Furth, 1938) or found no evidence for it (Medawar, 1940). The present study, being of a histological character, can offer no more than the suggestion that such a transformation would account for the very rapid replacement of large numbers of undoubted macrophages by equally many cells that are almost certainly fibroblasts, without the occurrence of many mitotic divisions.

SUMMARY.

An electron microscopical study, using ultra-thin sections, has been made of the pancreatitis of adult mice infected with a local strain (PL5) of Coxsackie virus.

The reaction is complex, is virtually confined to the acinar cells, and comprises 3 stages with some temporal overlap:

(1) *Degenerative.* This occupies approximately the first 48 hours. Pyknotic nuclear degeneration appears to be secondary to the cytoplasmic lesions, of which three main types can be distinguished:

(a) Focal lesions (laminated bodies) which begin by a local increase in vacuolation and extend, partly by breaking into pre-existing vacuolated cytoplasmic double membranes, and partly apparently by the deposition of new membranous material, to form complex concentric structures which occasionally show clumps of small particles near their centres. These particles are approximately 50-70 mμ in diameter, and some have clear centres. Mitochondria and zymogen granules intimately involved in laminated bodies show little change.

A proportion of laminated bodies later show terminal or central swellings of ruptured laminae, and appear to cast off these swollen masses into the intermembranous spaces.

(b) Diffuse cytoplasmic vacuolation (networks). The vacuoles are formed from pre-existing cytoplasmic double membranes. Often in a region of cytoplasm showing this change there are one or several superimposed focal laminations. Cells showing the network appearance are deeply basophilic in the visible-light microscope.

(c) Occasional large (5μ) dense inclusions, with small internal vacuoles. A large swollen granule, possibly an altered mitochondrion, has been seen in one of these inclusions.

(2) *Inflammatory.* The inflammatory reaction is almost exclusively mononuclear. At 24 hours many macrophages have begun to penetrate the acini, and at 48 hours they are very numerous among the degenerating acinar cells. They appear to be especially attracted to laminated bodies, but also ingest pyknotic nuclear fragments.

(3) *Fibrous replacement.* On and after the 4th day the macrophages filling the necrotic acini are rapidly replaced by fibroblasts. It is suggested that a transformation of macrophages to fibroblasts may occur.

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THE ADSORPTIVE CAPACITY OF SERUM PROTEINS IN RENAL INSUFFICIENCY

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It has been shown previously by one of us (Breyer, 1938) that the polarographic method is well suited to the investigation of adsorption/desorption processes involving organic dyes and proteins in solution. The method is based on the observation that the addition of protein to a dye solution lowers the polarographic diffusion current due to the dye because of its adsorption onto the protein. That the lowering of the current is due to dye-protein interaction and not to adsorption of protein onto the electrode, as suggested by Keilin (1948), follows both from polarographic experiments in which the dyestuff was displaced from the protein surface by more strongly adsorbed substances (Breyer, 1938), and from equilibrium dialysis experiments (Klotz, Walker and Pivan, 1946).

Breyer, Bauer and Radcliff (1953) and Breyer and Bauer (1953) have shown that for a given concentration of protein the lowering in diffusion current of the dye (i.e., the amount of dye adsorbed) is proportional to the total amount of dye added to the solution. The method, therefore, can be used as a quantitative measure of dye adsorption onto proteins in solution.

The addition of surface active agents, as e.g., bile salts, lipoids, urine, etc., to the dye-protein complex displaces some of the adsorbed dye from the protein surface and the diffusion current rises accordingly. Just as the decrease in diffusion current is a measure of the quantity of dye adsorbed, its increase is a measure of the amount of dye desorbed and hence of the power of the added surface active substance to displace dyestuff from the protein surface.

The addition of urine to the dye-serum complex displaces considerable amounts of dyestuff from the protein surface. It was, therefore, thought that in cases of renal insufficiency, where many substances which should pass into the urine are retained in the blood, the adsorptive power of the serum proteins might be significantly lowered. This, in fact, was shown to be the case, and it is the purpose of this paper to report the results obtained.

MATERIALS AND METHODS.

The following dyes were used: (i) 4'-dimethylamino-azobenzene-2-carboxylic acid (Methyl red). Commercial preparation (British Drug Houses Ltd., London) recrystallized from alcohol. (ii) 4'-hydroxy-azobenzene-4-carboxylic acid prepared by coupling diazotized

p-amino-benzoic acid with phenol. (iii) 2′-hydroxy-naphthalene-4-azo-benzoic acid prepared by coupling diazotized p-amino-benzoic acid with β-naphthol. (iv) 4′-dimethylamino-azobenzene-4-sulphonic acid (Methyl orange). Commercial preparation (British Drug Houses Ltd., London) recrystallized from alcohol. (v) 4′-hydroxy-azobenzene-4-sulphonic acid prepared by coupling diazotized sulphanilic acid with phenol. (vi) 2′-hydroxy-naphthalene-4-azo-benzene-sulphonic acid prepared by coupling diazotized sulphanilic acid with β-naphthol. (vii) 4-hydroxy-azobenzene prepared by coupling diazotized aniline with phenol. (viii) 4-amino-azobenzene (Aniline yellow). Commercial preparation (British Drug Houses Ltd., London) recrystallized from alcohol.

Dye solutions were prepared by triturating the requisite quantity of solid with a few drops of 2 N sodium hydroxide (acidic dyestuffs) or 2 N hydrochloric acid (basic dyestuffs) and diluting to volume with supporting electrolyte (most commonly buffer).

Universal buffer, pH 7·3, was prepared from acetic, phosphoric and boric acids according to the formula of Prideaux and Ward (1924).

The serum used in control experiments was normal horse serum, free of antiseptic, as supplied by the Commonwealth Serum Laboratories, Melbourne. When testing human specimens, either serum or oxalated plasma may be used as the presence of neither fibrinogen nor oxalate affects the binding capacity of the serum proteins.

Specimens of urine were brought to a pH of 7 by the addition of solid sodium bicarbonate.

Except where otherwise stated, samples were prepared by adding 1·0 ml. of serum or plasma to 2·5 ml. of $3{\cdot}3\times10^{-4}$ M dye solution and bringing the volume to 5·0 ml. by the addition of supporting electrolyte. When testing the dye displacing power of urine or other solutions, 1·0 ml. of the urine or solution was added to the dye-protein complex prepared as above in place of an equal volume of supporting electrolyte. All samples were thermostated at 25°±0·5° C., except where otherwise stated.

Details of the polarographic technique recommended for work with protein solutions are contained in a previous communication (Breyer and Radcliff, 1953).

RESULTS.

Since all azo-dyes give a polarographic step, the range of available dyestuffs is considerable. A series of experiments was conducted in order to determine the amounts of different dyes adsorbed under the same experimental conditions. The displacement of each dye by urine was also investigated. The results, shown in Table 1, indicate that Methyl red is the most suitable of the dyes examined. It combines a high diffusion current with a good adsorption/desorption ratio.

A further series of experiments was carried out to test the effect of various supporting electrolytes. The adsorption onto serum of Methyl red and its displacement by urine were measured in each instance. The results, listed in Table 2, show that the adsorption/desorption ratio is optimal in universal buffer, pH 7·3. Dissolved in this buffer Methyl red gave a clearly defined polarographic step with a half step potential of —0·36 volt *vs* the saturated calomel electrode. The reproducibility of individual results was ±3 p.c. and the diffusion current was proportional to the dye concentration within the same limits of accuracy (Fig. 1). Furthermore, under the given experimental conditions, the quantity of dye adsorbed was proportional to the total amount added within ±5 p.c. (Fig. 2).

TABLE 1.

Adsorption/desorption equilibria of different dyestuffs.

Dye	Dye step height in the absence of protein μA.	Dye adsorbed by serum p.c.	Dye adsorbed by serum in presence of urine p.c.	Adsorption/ Desorption Ratio
Methyl red	0·990	65	51	1·27
4′-hydroxy-azobenzene-4-carboxylic acid	0·461	51	39	1·31
2′-hydroxy-naphthalene-4-azo-benzoic-acid	1·12	72	66	1·09
Methyl orange	0·553	63	60	1·05
4′-hydroxy-azobenzene-4-sulphonic acid	0·639	52	45	1·16
2′-hydroxy-naphthalene-4-azo-benzene-sulphonic acid	1·12	76	71	1·07
4-hydroxy-azobenzene	0·805	65	61	1·07
Aniline yellow (saturated solution)	0·192	51	35	1·46

Dyestuff concentration $3{\cdot}3\times10^{-4}$ M in every case except Aniline yellow which is insufficiently soluble. The specimens of serum and urine were the same throughout. Temperature, 16° C.

TABLE 2.

Variation in adsorption/desorption of $3{\cdot}3\times10^{-4}$ M Methyl red with change of supporting electrolyte.

Supporting electrolyte	Dye step height in the absence of protein μA.	Dye adsorbed by serum p.c.	Dye adsorbed by serum in presence of urine p.c.	Adsorption/ Desorption Ratio
Universal buffer pH 7·3	1·21	57	47	1·21
Sat. sodium bicarbonate	0·821	49	42	1·17
0·05 M sodium bicarbonate	0·763	45	42	1·07
M ammonium acetate	1·22	29	25	1·16
0·25 M ammonium acetate	1·31	39	35	1·11
0·1 M phosphate buffer pH 7·3	0·979	38	36	1·06
0·05 M phosphate buffer pH 7·3	1·01	40	37	1·05

The specimens of serum and urine were the same throughout. Temperature, 21° C.

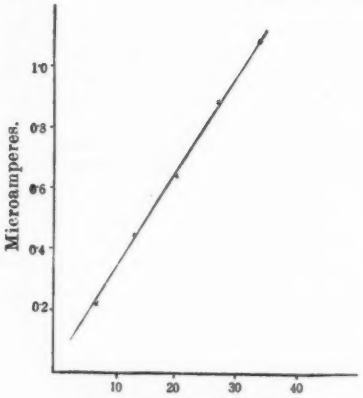

Fig. 1.

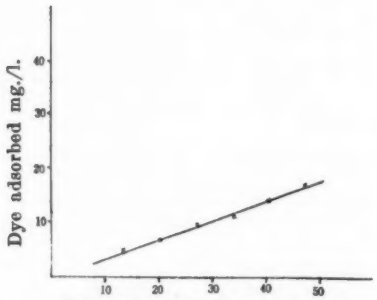

Fig. 2.

Fig. 1. Calibration curve of Methyl red in universal buffer, pH 7·3. Temperature, 23° C.

Fig. 2. Adsorption of Methyl red in universal buffer, pH 7·3, on to normal horse serum (serum dilution: 1 in 5). Temperature, 23° C.

TABLE 3.

Dye adsorbed by normal human sera.

Case Number	Sex	Age	Dye adsorbed p.c.
1	M	24	72
2	M	47	73
3	M	25	74
4	M	37	73
5	M	15	72
6	M	36	72
7	M	25	72
8	F	26	71
9	F	20	73
10	M	51	74
11	F	21	72
12	F	21	74
13	M	22	75
14	M	29	71

In Table 3 are given the figures showing the quantity of dye adsorbed by a number of normal human sera.

It follows that the binding capacity of normal serum for Methyl red is nearly constant and that its value, under the given experimental conditions, lies between 70 p.c. and 75 p.c. of the total amount of dye added.

The pathological sera examined in the course of these experiments were obtained from two groups of patients: those who were suffering from renal insufficiencies (Table 4) and those whose kidney function was unimpaired (Table 5).

TABLE 4.

Dye adsorbed by the sera of a number of patients suffering from renal insufficiency.

Case Number	Sex	Age	Diagnosis	Dye adsorbed p.c.	Blood Urea mg. p.c.	Blood proteins Total gm. p.c.	Albumin gm. p.c.	Globulin gm. p.c.
19	M	25	Chronic nephritis, cardio-renal failure	54	490	4·3	2·2	2·1
20	M	14	Acute haemorrhagic nephritis with oedema (20/12/51)	37	60	3·5	0·2	3·3
			(24/1/52)	46	15	3·8	1·4	2·4
22	F	5	Acute nephritis	71	25	6·0	4·1	1·9
25	M	31	Anuria due to sulfadiazine	60	245	7·6	4·3	3·3
26	M	37	Malignant hypertension *c.* subarachnoid haemorrhage	62	130	4·9	2·7	2·2
31	M	57	Anuria due to renal calculus	54	195	8·3	3·5	4·8
32	M	18	Acute pyelonephritis	70	50	8·2	4·5	3·7
33	M	44	Acute nephritis	68	25	6·6	4·2	2·4
34	F	41	Bilateral renal calculi, renal failure	62	130	7·8	3·6	4·2
35	M	61	Gastric carcinoma *c.* secondary liver metastases (Postoperative uraemia)	59	205	5·9	3·6	2·3
36	F	44	Acute nephritis	70	25	7·2	4·7	2·5
37	M	66	Carcinoma of bladder (Postoperative uraemia)	55	185	5·4	2·4	3·0
38	F	67	Hypertensive nephrosclerosis *c.* uraemia	63	95	6·5	3·7	2·8
39	M	79	Essential hypertension, pyelonephritis *c* pre-uraemia	64	120	6·8	3·8	3·0
40	F	62	Chronic nephritis and hypertension, renal failure	61	210	5·7	4·1	1·6
41	F	78	Chronic nephritis, uraemia, refractory anaemia	47	160	7·4	3·4	4·0
42	F	37	Malignant hypertension *c.* uraemia	66	160	6·7	3·9	2·8
43	F	55	Typhi murium dysentery *c* pre-uraemia, hypostatic pneumonia	61	305	5·0	3·3	1·7
44	M	71	Carcinoma of prostate. Died 12 hrs. after hospital admission	60	90	5·1	2·6	2·5
45	F	82	Acute enteritis, renal failure	54	380	5·6	3·4	2·2
47	M	58	Tuberculosis of kidney and bladder	70	90	7·4	4·6	2·8
48	F	43	Chronic nephritis	63	90	6·4	4·1	2·3
49	M	48	Malignant hypertension, cardio-renal failure	60	500	6·4	4·7	1·7
50	F	76	Lobar pneumonia, *c.* uraemia	60	85	5·0	3·3	1·7
51	F	70	Congestive cardiac failure, coronary sclerosis	71	100	6·2	3·6	2·6
52	M	66	Congestive cardiac failure, myocardial degeneration	60	105	5·0	2·5	2·5
54	F	67	Acute tubular necrosis, renal failure, anuria	54	240	5·4	2·8	2·6
55	F	68	Carcinoma of pelvic sigmoid colon. (24/6/52)	56	120	6·5	3·6	2·9
			Diabetes mellitus (Postoperative uraemia) (27/7/52)	55	20	4·1	2·0	2·1
56	M	69	Essential hypertension, cardio-renal failure	62	295	6·0	3·9	2·1
57	F	63	Chronic nephritis, pre-uraemia, secondary anaemia	58	60	5·9	2·5	3·4
63	F	48	Chronic nephritis, renal failure	45	550	4·7	3·0	1·7

TABLE 5.

Dye adsorbed by the sera of a number of patients whose kidney function was unimpaired.

Case Number	Sex	Age	Diagnosis	Dye adsorbed p.c.	Blood Urea mg. p.c.	Blood proteins Total gm. p.c.	Albumin gm. p.c.	Globulin gm. p.c.
15	F	65	Fracture of right femur	68	—	6·7	3·7	3·0
16	F	30	Lacerated wound of scalp	68	—	5·5	3·2	2·3
17	M	18	Thrombosed peri-anal haematoma	70	—	7·2	4·3	2·9
18	M	60	Left chronic infra-patellar bursitis	69	—	6·6	2·8	3·8
23	M	59	Carcinoma of prostate, acute urinary retention	66	15	5·8	3·0	2·8
27	F	51	Carcinoma of ovary *c.* abdominal metastases	70	—	7·8	4·8	3·0
28	F	71	Subacute obstruction of large bowel	67	—	6·4	3·2	3·2
29	F	53	Hepatitis	69	20	7·5	4·5	3·0
30	F	10	Scarlet fever (exanthematic stage)	71	—	8·1	4·0	4·1
46	M	67	Adenoma of prostate	71	35	7·7	5·0	2·7
58	M	62	Carcinoma of stomach *c.* abdominal metastases	68	20	5·3	3·0	2·3
59	F	26	Asthma	67	20	6·3	4·6	1·7
60	M	18	Encephalitis	70	35	5·9	3·7	2·2
61	F	57	Cirrhosis of the liver	52	20	6·7	2·3	4·4
62	M	65	Carcinoma of hepatic flexure with obstruction	65	25	5·8	3·3	2·5

It is evident from Table 4 that in all cases of chronic renal insufficiency examined, the adsorptive capacity of the serum was less than 65 p.c. All sera were analysed for protein content to see whether the polarographic results obtained were not due to changes in protein concentration. It will be seen that there was no relationship between protein concentration and polarographic results, with the possible exception of Case Number 20, where the low adsorption value was most probably due to an abnormally small serum albumin level.

TABLE 6.

Desorptive capacity of different urines diluted to a creatinine level of 100 mg. p.c.

Specimen identification	Dye adsorbed in absence of urine p.c.	Dye adsorbed in presence of urine p.c.
1	55	45
2	54	40
3	55	36
4	55	39
5	55	38

TABLE 7.

Desorptive capacity of the major constituents of normal urine and of a number of organic compounds, allied in structure to the dye molecule.

Desorbant	Dye adsorbed p.c.
Buffer (control)	55
Urine (random specimen)	37
Dialysed urine (same specimen)	49
Urea (2 gm. p.c.)	58
Uric acid (50 mg. p.c.)	59
Hippuric acid (50 mg. p.c.)	56
Creatinine (100 mg. p.c.)	56
Ammonia (51 mg. p.c.)	58
Solution of inorganic salts as normally found in urine*	49
0·02 M p-amino-benzoic acid	50
0·02 M sulphanilic acid	43
0·02 M phenol	47
0·02 M di-sodium-phenyl-phosphate	43

* This salt solution was made up as follows:

KCl 2·86 gm./l.	Na_2SO_4 3·26 gm./l.
$CaCl_2$ 0·55 gm./l.	$Na_2HPO_4 \cdot 12H_2O$ 1·26 gm./l.
$MgCl_2.6H_2O$ 0·84 gm./l.	$NaH_2PO_4 \cdot 2H_2O$ 4·95 gm./l.
NH_4Cl 1·19 gm./l.	NaCl 5·45 gm./l.

It seems that cases of acute nephritis yield negative results (Cases Number 22, 32, 33 and 36). Congestive cardiac failure with impaired renal elimination may or may not give a positive result. Thus, for instance, in cases Number 51 and 52 the adsorptive capacity of the serum proteins was normal in one and low in the other case, although the blood urea values were practically identical.

The only instance in which a dye adsorption of less than 65 p.c. was obtained for the serum from patients suffering from pathological conditions other than renal insufficiency (Table 5), was in a case of cirrhosis of the liver (Case Number 61).

Table 6 shows the desorptive capacity of different urines diluted to the same creatinine concentration. It is evident that the desorptive capacity varies greatly.

Finally, experiments were carried out to test the desorptive power of some of the major constituents of normal urine (Table 7). It can be seen that none of them is responsible for the desorption.

A number of organic compounds, allied in structure to the dye molecule, was also tested for their displacing power. The considerable effect of phenol and phenyl phosphate as well as of the sulphonic acid group is particularly noteworthy.

DISCUSSION.

It follows from the results reported in the previous section that in cases of renal insufficiency the adsorptive capacity of serum is lower than normal. The most feasible explanation is that a substance, normally excreted in the urine, is retained in the blood and competes with the dye for binding sites on the protein surface. This is borne out by the fact that, by the addition of normal urine to the dye-protein complex, protein bound dyestuff is set free. Further support for this view can be derived from the observations that purely mechanical blockage of the renal tubules, as in the case of sulfadiazine anuria (Case Number 25), as well as anuria due to renal calculus (Cases Number 31 and 34), leads to a lowering of the adsorptive capacity of serum proteins.

The results also show that none of the major nitrogenous constituents of urine has significant displacing power. The inorganic salts present in urine have a lowering effect on the dyestuff binding power of serum, to a much smaller extent, however, than urine itself. That salts are only partly responsible was further shown by dialysis experiments: the dialysed urine still retained considerable dye displacing power (Table 7). This points to the presence in urine of an undialysable substance which has a desorptive effect on the dye-protein complex. That salts play only a minor part in diminishing the binding capacity of serum proteins, follows also from the fact that the salt concentration in all pathological sera examined was found normal, although the desorptive capacity varied greatly.

It seems significant that adsorption values do not run parallel to blood urea concentrations (Table 4). This agrees with the above observation that urea has no effect upon the adsorption/desorption equilibrium of the dyestuff. It is conceivable that the responsible factor is either a phenol or indole derivative as, e.g., phenyl sulphate or indoxyl sulphate, since it is known that the

concentration of these substances in blood increases in uraemia (c.f. for instance, Kolmer, 1943). Results shown in Table 7 also point to the considerable desorptive activity of substances containing similar groups or having an allied configuration to those mentioned above.

Future research will have the object of isolating the factor responsible for the phenomenon observed.

SUMMARY.

The binding capacity of serum proteins for Methyl red was studied polarographically. It was found that in cases of renal insufficiency the binding power was considerably lowered. It seems probable that the phenomenon is due to the presence in blood of a substance normally passed into the urine. The possible nature of this substance is discussed.

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THE SCREENING OF 117 CULTURES OF FUNGI AND YEASTS FOR ANTIVIRAL AND ANTIBACTERIOPHAGE ACTIVITY

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The inadequacies of knowledge concerning viral multiplication have contributed in no small way towards the rather haphazard screening of many substances for antiviral properties. Furthermore, technical difficulties have, of necessity, imposed strict limitation not only upon the number of agents examined but also upon the spectrum of viruses available for test purposes. Despite such discouraging factors, however, a number of biological materials capable of modifying at different stages the infectious cycles of plant (Gupta and Price, 1950), animal (Horsfall, 1950; Ginsberg, 1952; Gropé *et al.*, 1951), or bacterial (Asheshov *et al.*, 1949; Hall *et al.*, 1951) viruses have been recorded. Of considerable interest has been a recent report by Shope (1953) that injection of an extract from *Penicillium funiculosum* exerted a sparing effect upon mice infected with the viruses of Semliki forest, Columbia SK or swine influenza. Some insight into the mechanism of this modifying effect was provided by the reduced antibody titres in recovered animals, a finding suggestive of an impairment in the production of fully synthesized virus. A similar observation had previously been recorded by Powell *et al.* (1952) for mice protected against the MM virus by a filtrate from an unidentified Penicillium culture.

The present work largely originated from the development of a simple tissue culture method for infectivity titrations with the viruses of Western equine encephalomyelitis and of Newcastle disease (Fastier, 1954). Together with conventional chick embryo screening tests using the PR8 and LEE strains of influenza type A and B viruses respectively, this technique has been utilized in the investigation for possible antiviral activity of crude filtrates from 117 cultures of saprophytic and parasitic fungi. At the same time each extract was examined for similar properties against bacteriophages for *Sh. paradysenteriae* (type I and type II), *Sh. sonnei* and *Staph. aureus*. Conventional antibacterial screening procedures against *Esch. coli* and *Staph. aureus* were also included.

Materials and Methods.

Viruses.

The origin and passage history of the PR8 strain of influenza type A virus, the Olitsky strain of Western equine encephalomyelitis (WEE) and the California strain of Newcastle disease virus (NDV) have been described elsewhere (Fastier, 1952; 1954). Stock glycerinated

allantoic fluid pools were prepared from the 16th and 9th passages respectively of the PR8 and NDV strains. A glycerinated 10 p.c. suspension of embryo body infected with the WEE virus (8th egg passage) was made in nutrient broth. The LEE strain of influenza type B virus was obtained in 1949 from Dr. E. H. Lennette, State Virus and Rickettsial Laboratory, Berkeley, California. The preparations glycerinated for storage during the present experiments were all derived from the 15th allantoic passage. All viruses were stored in small volumes at −30° C. Employing the titration methods described below, these suspensions regularly yielded an EID_{50} of $10^{-8.5}$ (PR8 virus) and $10^{-9.0}$ (LEE virus). The WEE and NDV strains possessed a chick embyro yolk sac LD_{50} of $10^{-8.2}$ and $10^{-8.5}$ respectively, the corresponding tissue culture end-point (TC_{50}) being $10^{-8.5}$ in both instances.

Virus infectivity titrations.

Both the LEE and PR8 strains were routinely titrated by allantoic inoculation (0·1 ml.) of at least four 11-day-old chick embryos per ten-fold dilution. Following incubation at 36° C. for 48 hours, the individual allantoic fluids were harvested and "spotted" with one drop each of a 20 p.c. fowl red cell suspension. The EID_{50} was subsequently calculated by the Reed and Muench formula (Reed and Muench, 1938) from the presence or absence of haemagglutination.

An *in vitro* technique, reported elsewhere (Fastier, 1954), was employed for infectivity titrations with NDV and the WEE virus. Briefly, this consisted of incubating 0·5 ml. of each serial ten-fold virus dilution (in Simms ×7 solution) with an equal volume of the same diluent containing ten small fragments of 9-11 day-old chick embryo skeletal muscle. Four such cultures were prepared in 15×150 mm. test tubes for each virus dilution. After incubation at 37° C. for 48 hours (WEE) or for 72 hours (NDV), the supernates were removed and the residual tissue clotted in a mixture consisting of one drop each of heparinized fowl plasma and 50 p.c. chick embryo extract. The cultures were reincubated for a further 48 hours and examined at a magnification of 30 diameters to determine the extent of fibroblast outgrowth from each explant. As described elsewhere, this response could be divided into three categories: (a) maximal growth comparable to control cultures without virus, indicating absence of virus in inoculum; (b) complete absence of growth, indicating presence of virus in inoculum, and (c) reduced corona of growth, the intermediate phase, occasionally exhibited by cultures inoculated with limiting dilutions of virus. The results from these latter tubes were included within group (b). The tissue culture end-point (TC_{50}) was the virus dilution calculated to give complete or almost complete suppression of growth in one-half the number of inoculated tubes.

Preparation of yeast and fungal extracts.

All cultures were propagated in a fluid medium of the following composition:

Cornmeal, 40 gm.
Dextrose, 20 gm.
Bacto beef extract, 4 gm.
Distilled water, 1,000 ml.

The above ingredients were heated for 1 hour at 60° C. followed by steaming for 30 minutes. The fluid was then decanted, clarified by passage through a sintered glass filter and sterilized by steaming on two successive days. The medium (final pH 7·0) was dispensed in 40 ml. volumes in flat-sided 8 oz. bottles which, after inoculation, were incubated in a horizontal position. As a routine procedure two such vessels were inseminated from each stock culture, one being harvested at 12 days and the duplicate after 28 days incubation in the dark at room temperature (20-21° C.). The fluid phase of each culture was removed and

the cellular elements ground with sand in 4 ml. of 0·2 M phosphate buffer, pH 7·2. During this time the material was subjected to two slow freezings and thawings. When completed, the corresponding supernates and deposits were combined, centrifuged at 2,500 r.p.m. for 10 minutes, and the resulting supernates stored at —30° C. Prior to use, both the 12-day and 28-day harvests were combined and the pH adjusted where necessary to 7·2 with 0·5 M Na_2HPO_4 in the presence of phenol red indicator. Each extract was then sterilized by passage through a Berkefeld N filter and refrozen. Where inadequate fungal growth had occurred by the 12th day of incubation, both cultures were processed simultaneously after one month at room temperature.

Screening for toxicity.

Preliminary experiments with 11-day-old chick embryos showed that no ill-effects followed two daily intra-allantoic inoculations (0·75 ml. each) with a number of undiluted culture filtrates. Separate toxicity tests were therefore dispensed with and reliance placed in the absence of an undue number of embryo deaths at time of harvest. Within the experimental period neither influenza strain was lethal for the developing chick. Under tissue culture conditions however, many filtrates, even when mixed with an equal volume of diluent, proved intensely cytotoxic. As a routine procedure, therefore, separate standard suspended tissue cultures (0·5 ml.) were incubated for 48 hours at 37° C. with an equal volume of fungal filtrate diluted 1:5 and 1:10 in Simms' solution. The supernates were then removed and two drops each of embryo extract, the corresponding dilution of filtrate, and heparinized plasma added to induce clotting. After a further 48 hours' incubation the toxicity end-point was taken as the dilution of filtrate in which fibroblast outgrowth was unimpaired.

Screening for antiviral properties.

According to the nature of the test virus the following procedures were employed. In the case of PR8 virus eight 10-day-old chick embryos were inoculated intra-allantoically with 0·75 ml. of each undiluted filtrate. The eggs were reincubated at 36° C. for a period of two hours, during which time they were rotated at 15 r.p.h. for 15 minutes. All embryos were then inoculated by the allantoic route with 0·1 ml. of PR8 virus diluted to contain 10 EID_{50}. Following reincubation for 24 hours each egg received by the same route a further 0·75 ml. of filtrate and, after a total incubation time of 48 hours, the allantoic fluids were individually harvested and "spotted" with fowl erythrocytes to determine the presence or absence of virus. Where haemagglutination occurred the corresponding harvests from each group were pooled and titrated by the standard Salk pattern technique. A similar procedure was followed with the LEE strain of influenza type B virus.

With WEE virus standard tissue, suspensions (0·5 ml.) were inoculated with 0·25 ml. of the lowest non-toxic dilution of fungal filtrate, followed two hours later with 0·25 ml. of virus diluted to contain 20 TC_{50}. Four tubes were employed for each filtrate. After incubation at 37° C. for 48 hours, the growth potential of all tissue fragments was determined as described in the toxicity determinations. A similar procedure was followed with NDV, with the one exception that initial contact between virus, fungal filtrate and tissue was prolonged to 72 hours. Prevention of the cytopathogenic effect exhibited by either virus was accepted as the criterion of a potentially active filtrate.

Screening for antibacteriophage activity.

Bacteriophages for *Staph. aureus, Sh. paradysenteriae* type I (Flexner V), *Sh. paradysenteriae* type II (Flexner W) and *Sh. sonnei* were each propagated in nutrient broth upon their corresponding bacterial hosts. When satisfactory plaque counts were obtained, preparations were Seitz filtered and stored at 4° C. For testing fungal or yeast extracts a nutrient

agar plate was flooded with a mixture of bacteria and a dilution of homologous bacteriophage sufficient to give almost complete lysis. After drying, filter paper discs (8·0 mm. diameter) saturated with each undiluted filtrate, were laid upon the surface and the plates incubated overnight at 37° C. (Asheshov *et. al.*, 1949). The results were recorded as either inhibition or no inhibition of the lytic effect.

Screening for antibacterial activity.

All filtrates were tested in the undiluted state against *Staph. aureus* (Oxford strain) and a recently isolated culture of *Esch. coli*. The "cup" technique was used throughout, each hole (7·0 mm. diameter) being made in nutrient agar previously spread with a broth culture of the appropriate organism. The presence or absence of bacterial inhibition was noted after overnight incubation at 37° C.

List of cultures examined.

NCTC=National Collection of Type Cultures, Imperial Mycological Institute, Kew.
Delft=Centraalbureau voor Schimmelcultures, Delft, Holland.

Cultures unaccompanied by specific identification numbers represent strains isolated within New Zealand.

Mucor mucedo (NCTC 26440), *Absidia butleri* (NCTC 37126), *Endomycopsis fibuliger* (NCTC 1874), *Saccharomyces cerevisiae* (NCTC 381), *Sacc. acidifaciens, Pichia membranifaciens* (NCTC 479), *Pichia fermentans, Hansenula anomala* (NCTC 474), *Debaryomyces marama, Deb. kloeckeri* (Delft), *Hanseniospora valbyensis* (NCTC 478), *Saccharomycodes ludwigii* (Delft), *Nematospora coryli, Schwanniomyces occidentalis* (NCTC 476), *Sporobolomyces roseus, Cryptococcus laurentii, Crypt. diffluens, Brettanomyces bruxellensis* (Delft), *Candida albicans, Cand. utilis, Cand. krusei, Torulopsis glabrata, Rhodotorula glutinis, Rhod. mucilaginosa.*

Phoma lingam, Sporotrichum bombycinum (NCTC 23280), *Cephalosporium asteris, Trichoderma viride* (NCTC 16198), *Trich. viride* (NCTC 24039), *Verticillium dahliae* (NCTC 17460), *Verticillium* sp., *Botrytis allii, Bot. tulipae, Cladosporium fulvum, Helminthosporium teres* (NCTC 21195), *Helminthosporium* sp., *Alternaria brassicae, Alternaria* sp., *Fusarium* sp. 1, *Fusarium* sp. 2, *Fus. sambucinum, Fus. graminearum, Fus. moniliforme.*

Aspergillus candidus, Asp. ustus, Asp. niger (Mulder's strain), *Asp. niger, Asp. flavus* (London School of Hygiene, A.4), *Asp. flavus* (NRRL 524), *Asp. flavus* (London School of Hygiene B.B.67), *Asp. flavus* (London School of Hygiene, A.1), *Asp. flavus, Asp. versicolor, Asp. oryzae* (NRRL 460), *Asp. fumigatus, Gliocladium fimbriatum* (NCTC 399191), *Gliocladium* sp.

Penicillium commune, P. luteum, P. italicum, P. restrictum, P. notatum (MRC 3568), *P. notatum* (USA 832), *P. brevi-compactum* (USA 1249 B21), *P. brevi-compactum, P. chrysogenum, P. chrysogenum* (Merck X1612), *P. oxalicum, P. chermisinum, P. canescens, P. waksmani, P. velutinum, P. jenseni, P. raciborskii, P. frequentans, P. vinaceum, P. atramentosum, P. albidum, P. steckii, P. martensii, P. phoeniceum, P. corymbiferum, P. digitatum.*

Trichophyton sulphureum, Trich. mentagrophytes, Trich. rubrum, Microsporum gypseum, Mic. canis, Mic. deformans (n.s.), *Epidermophyton floccosum.*

Rhizoctonia solani (wheat), *Rhiz. solani* (potato), *Sclerotinia rolfsii, Sclerotinia sclerotiorum* 1, *Sclerotinia sclerotiorium* 2, *Physalospora cydoniae, Didymella lycopersici, Dothiorella* sp., *Heteropatella valtellinensis, Mortierella pusilla* (NCTC 14780), *Stereum purpureum, Cercosporella herpotrichoides Phialea temulenta, Septoria pisi, Sept. divaricata, Theilaviopsis basicola* (NCTC 36482), *Theilaviopsis* sp., *Papularia* sp., *Poecilomyces* sp., *Dicoccum* sp., *Pestalozzia* sp., *Epicoccum* sp., *Rhinocladium* sp., *Nectria galagena, Stemphylium* sp., *Pleospora* sp., *Pullularia* sp., *Geomyces* sp.

EXPERIMENTAL.

When assayed by the above methods none of the 117 fungus or yeast cultures was found to inhibit virus growth. In all instances where the chick embryo protection test was employed, allantoic fluids, when harvested from living embryos after a total volume of 1·5 ml. of each extract had been administered during virus infection, possessed influenza haemagglutinin titres comparable to those eggs inoculated with virus alone. Considering the diverse nature of the fungi, it was surprising to find comparatively few embryo deaths directly attributable to filtrate toxicity. Although in most instances such filtrates were inoculated in the undiluted state, where a mortality rate of 50 p.c. or greater was encountered (*Penicillium restrictum, P. martensii, Fusarium sambucinum, F. graminearum*) the experiment was repeated with the lowest non-toxic dilution. On the other hand, the natural cytotoxicity exhibited by extracts under tissue culture conditions necessitated the majority to be screened at a 1:10 dilution. The four strains with high embryo mortality rates, together with *Trichoderma viride* and *Gliocladium fimbriatum*, were notable exceptions in requiring dilutions of 1:60 and 1:100 before use. Irrespective of whether tissue cultures or fertile eggs were employed, the amount of each fungal extract administered closely approximated the maximum tolerated dose, since marked cytotoxicity or embryo death invariably attended inoculation of greater volumes.

At regular intervals titrations were made on the virus dilutions used for infecting either chick embryos or tissue cultures. As such results showed the number of EID_{50} or TC_{50} contained therein to lie between 10 and 20, it was apparent that an excessive virus challenge was not responsible for the inability to demonstrate any potential antiviral activity in each extract tested.

TABLE 1.

Fungal cultures exhibiting antibacterial activity.

Culture	*Staph. aureus*	*Esch. coli*
Gliocladium fimbriatum (NCTC 399191)	+++*	—
Gliocladium sp.	+	—
Trichoderma viride (NCTC 16198)	+	—
Trichoderma viride (NCTC 24039)	++	—
Verticillium sp.	+	—
Dicoccum sp.	+	—
Phoma lingam	+++	—
Fusarium moniliforme	+	—
Penicillium atramentosum	++	—
P. notatum (MRC 3568)	+++	—
P. notatum (USA 832)	+++	—
P. martensii	+++	+++
P. chrysogenum	+++	—
Aspergillus versicolor	+	—
Asp. oryzae	++	—

Filtrates examined from 12 and 28-day fluid cultures at 20° C.
*Inhibition zone (excluding 'cup'), 2– 7 mm. diam.=+
8–20 mm. diam.=++
over 20 mm. diam.=+++

Since a number of fungal cultures employed in the present work were specifically obtained for their antibiotic-producing ability, it was not surprising that such activity could be demonstrated during both antibacteriophage and antibacterial assays. Where the latter phenomenon was encountered while testing for antibacteriophage action, the appearance of plaques within the zone of bacterial growth immediately adjacent to the corona of inhibition was indicative of the absence of such a principle. Of the 117 fungus and yeast extracts tested separately by the paper disc method against four bacteriophages, none prevented plaque formation.

Antibacterial action was consistently shown in 28-day cornmeal broth cultures from the fungi listed in Table 1. However, since activity in most of these filtrates could be ascribed to known antibiotics (Florey *et al.*, 1949), and secondly, since none exhibited antiviral properties, no attempt was made to characterize the responsible substances.

SUMMARY.

The present work records the results of a systematic examination for the presence of antibacteriophage and antiviral activity among 117 cultures of both yeasts and fungi. From the outset it was realised that the handling of such a survey would necessitate arbitrary standardization, not only of the factors governing fungal growth, but also of the techniques applied to the actual screening of such agents for antibiotic activity. It must, therefore, be emphasized that the restricted experimental conditions employed above by no means exclude any of the fungal isolates from being potential antibiotic-producers under different environmental circumstances.

Neither haemagglutinin production in the chick embryo by the PR8 or LEE strains of influenza virus, nor the cytopathogenic changes in tissue culture which accompanied growth on the viruses of Western equine encephalomyelitis or of Newcastle disease were prevented by pre-treatment of either tissue system with fluid extracts of the fungi under test. Such extracts likewise failed to inhibit plaque formation when added to separate combinations of bacteriophages for *Staph. aureus, Sh. sonnei, Sh. paradysenteriae* types I and II and their corresponding bacterial hosts.

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STUDIES WITH RADIO-IODINATED INSULIN

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These experiments were designed to study several aspects of insulin physiology using crystalline insulin labelled with I^{131}. Labelled insulin has been prepared by direct radio-iodination of the protein hormone (Ferrebee *et al.*, 1951). We have used a modification of their technique. All our animals received 50 mg. subcutaneously of NaI prior to the experimental period. Preliminary experiments showed that this blocked the subsequent production of radioactive thyroxine from NaI^{131}. Consequently, in these animals the protein bound radioactive iodine (PBI^{131}) content of the blood and tissues was not influenced by thyroid activity.

Our main interest has been to determine the disappearance rate of PBI^{131} from the blood and its uptake by various tissues with different techniques of injection.

METHODS.

Preparation of labelled insulin.

5 mg. crystalline insulin were dissolved in 0·1 ml. N/10 HCl and 2·0 ml. M/15 phosphate buffer added to bring the pH to 8·0. To this solution 5 mC. of I^{131}, treated for the removal of sulphite, were added. The mixture was allowed to stand at room temperature in a sealed vessel for eight hours. It was then transferred to a cellophane bag and dialysed against successive changes of M/300 $NaHCO_3$ containing 0·1 p.c. tricresol for 60 hours at 4° C.

PBI^{131} was determined in tissues by macerating weighed quantities (approx. 1 gm.) in 10 ml. 1 p.c. trichloracetic acid. Protein was precipitated by increasing the trichloracetic acid concentration to 5 p.c. and centrifuging. The precipitate was suspended in water and reprecipitated in a similar manner and centrifuged again. This washing process was repeated twice more, the precipitate finally dissolved in 15 p.c. sodium hydroxide solution and assayed, using M6 or M12[2] liquid G.-M. tubes as described by Veall (1948).

Blood samples were prepared as described for tissues after maceration.

Blood glucose was determined by the method of Prokhovnik and Nelson, 1953.

Rats weighing 250-300 gm. and mice weighing 30-35 gm. were used throughout these experiments.

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[1]Working with a full-time grant from the National Health and Medical Research Council.

[2]Manufactured by 20th Century Electronics Ltd., England.

Labelled insulin was given by a single quick injection into the tail vein and as a prolonged continuous infusion into the inferior vena cava (I.V.C.) and portal vein. All rats receiving labelled insulin by continuous infusion were anaesthetized with pentobarbital sodium and the body temperature maintained at 36° C. in a warm chamber.

RESULTS AND DISCUSSION.

Biological activity of the labelled insulin.

Fig. 1 shows the mean blood glucose curve of 13 rabbits following 0·03 mg. of standard crystalline insulin. Four days later the same group of rabbits was given 0·03 mg. of the labelled insulin. The animals were fasted for 24 hours prior to each experiment. These curves show that the whole procedure, including iodination, pH adjustments and dialyses, has resulted in no appreciable loss of biological activity.

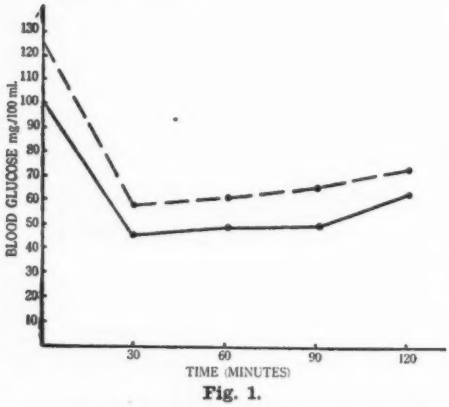

Fig. 1.

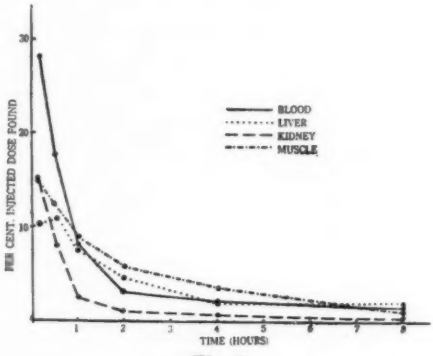

Fig. 2.

Fig. 1. Mean blood glucose curve of 13 animals following 0·03 mg. of insulin. —— crystalline insulin; - - - - iodinated insulin.

Fig. 2. The per cent. of the injected dose of PBI^{131} found in various organs and its rate of disappearance after a single injection of 2 units of labelled insulin. Each point represents the mean result from 3 rats.

Many other proteins have been labelled by direct radio-iodination without loss of biological activity. Thus there is a retention of biological activity of prolactin after labelling with an average of two iodine atoms per protein molecule (Cox, 1951). Similar results have been obtained with labelled ACTH (Ferrebee, 1951).

If the protein is labelled with less than one atom of I^{131} per protein molecule, then biological assay of the material becomes a less reliable index of the biological activity of the labelled molecules. Assuming the molecular weight of insulin to be 12,000 and that our sample of insulin is pure, only 16 p.c. of the protein molecules have been labelled.

Uptake by various organs and disappearance rate from blood.

Table 1 shows the PBI^{131} content of blood, liver, muscle and kidney at various times following a single quick injection of 2 units of insulin labelled with 1·6 μC. I^{131} into the tail vein. The results are expressed in three ways: The concentration of PBI^{131} per gm. of tissue, the organ/blood ratio, and the per cent. of the injected dose in that organ, the last being presented graphically in Fig. 2.

TABLE 1.

PBI^{131} content of blood, liver, muscle and kidney at various times following a single quick intravenous injection of 2 units of insulin labelled with 1·6 μC. I^{131}. The values for each time are the mean result from 3 rats.

Time (hours)	Blood		Liver		
	Conc. protein bound I^{131} (mμ curie/ml.)	P.c. injected dose in whole organ	Conc. protein bound I^{131} (mμ curie/gm.)	P.c. injected dose in whole organ	[Organ]/[blood]
1/6	15·3	28·0	13·3	10·2	0·87
1/2	9·5	17·4	14·5	11·4	1·53
1	4·4	8·2	10·1	7·6	2·3
2	1·6	2·9	6·4	4·6	4·0
4	1·5	2·7	3·4	2·5	2·3
8	0·8	1·4	2·5	1·7	3·1

Time (hours)	Kidney			Muscle		
	Conc. protein bound I^{131} (mμ curie/gm.)	P.c. injected dose in whole organ	[Organ]/[blood]	Conc. protein bound I^{131} (mμ curie/gm.)	P.c. injected dose in whole organ	[Organ]/[blood]
1/6	135	14·7	8·8	2·2	14·5	0·14
1/2	85	8·0	8·9	1·9	11·9	0·20
1	30	2·5	7·0	1·3	9·0	0·30
2	14	0·9	8·8	0·9	6·0	0·56
4	12	0·7	8·0	0·7	3·9	0·47
8	6	0·3	7·5	0·2	1·0	0·25

The concentration of PBI^{131} in the blood and kidney decreases rapidly and at the same rate. Consequently, the kidney/blood ratio remains fairly constant at 7 to 8·9. Because of this large concentration in the kidney it was thought worthwhile to determine how much PBI^{131} was excreted in the urine in the first hour. After intravenous administration, less than 1 p.c. of the injected dose was excreted in the urine in that time. The rapid disappearance of PBI^{131} from the blood cannot be explained by loss in the urine.

The rate of decay in the liver and muscle is much slower than in blood. Consequently the organ/blood ratio gradually increases over the first 2 hours.

It may be noted that the liver is the only tissue in which the concentration of PBI^{131} actually increases after the first ten minutes.

The PBI^{131} content was also determined in several other tissues. Of particular interest was the absence of PBI^{131} in the brain. This may lend support to the concept that insulin has no direct effect on brain metabolism.

To estimate the rate of destruction of insulin by the rat, the total amount of labelled insulin found at various times after injection was calculated from the organs investigated.

The disappearance rate of PBI^{131} following a single intravenous injection into mice was also determined. The values for mice were taken from the whole animal without skin. These results are shown in Fig. 3. To get the value for Time 0 the mice were killed, placed in 1 p.c. trichloracetic acid and blended within 30 seconds of the injection. Under these conditions 86·2 p.c. of the injected dose was recovered. Thereafter the amount of PBI^{131} which could be recovered rapidly decreased. The disappearance curve for the mouse and rat are very similar.

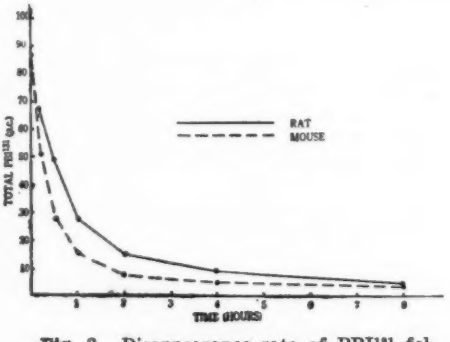

Fig. 3. Disappearance rate of PBI^{131} following a single intravenous injection. The values for mice are taken from whole animals while those for rats are the sum of the blood, liver, kidney and muscle. Each point represents the mean result from 3 animals.

Sonenberg (1952) has discussed the difficulties in the interpretation of results obtained with labelled proteins, especially with regard to the disappearance rate in blood and localization in particular tissues. Our studies with labelled insulin show different patterns from other labelled hormones and bovine serum albumen (Sonenberg, *et al.*, 1951a, 1951b). Fig. 4 illustrates the differences in behaviour of some radio-iodinated proteins. The values for prolactin and bovine serum albumen have been taken from results published by Sonenberg *et al.* (1951a, 1951b). These results at least indicate that iodinated insulin behaves differently from other labelled proteins, but of course does not prove specificity.

The organ/blood ratio and its change with time may be of some value in suggesting specific binding or penetration into cells. However, the high concentration in the kidney and liver may be due to the action of the reticulo-endothelial system on a foreign protein. Labelled bovine serum albumen does not concentrate to nearly the same extent in the liver or kidney as does labelled insulin.

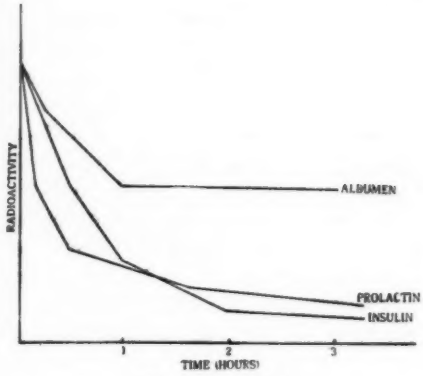

Fig. 4A. Blood.

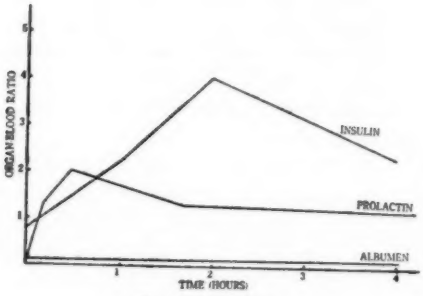

Fig. 4B. Liver.

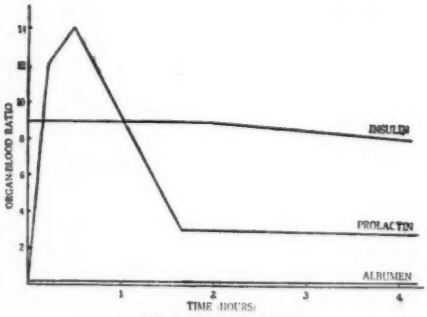

Fig. 4C. Kidney.

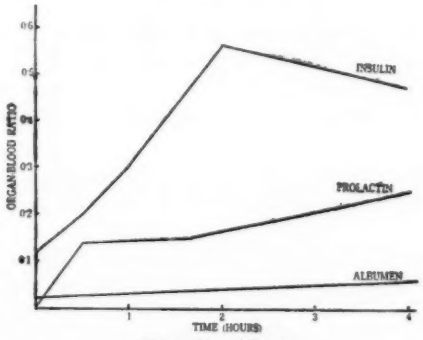

Fig. 4D. Muscle.

Fig. 4. Fig. 4A shows the disappearance rate pattern of labelled albumen, prolactin and insulin.

The change in organ/blood ratio at various times is shown in Fig. 4B (liver), Fig. 4C (kidney) and Fig. 4D (muscle).

Continuous infusion into I.V.C. and portal vein.

The same dose of labelled insulin (2 units of insulin labelled with 1·6 mC. of I^{131}) was given by continuous infusion into 2 groups of rats. In the first group the insulin was given via the I.V.C. and the second group into the portal vein. The arterial and portal blood samples were taken after 1 hour and whilst the infusion was still in progress. The tissue samples were taken at the same time. (The rats were then perfused with warm Ringer's solution and further samples removed. This will be discussed in the next section.) The results are represented graphically in Fig. 5. When labelled insulin was given via the portal vein the concentration of PBI^{131} in the arterial blood was 16 p.c. lower, the portal blood 25 p.c. and liver 50 p.c. higher than when the same dose was

infused into the I.V.C. There are two reasons for the higher concentration of PBI^{131} in the portal blood and liver following intraportal infusion. The labelled insulin goes directly to the liver and is therefore not first diluted in the whole blood volume and removed by kidney and muscles. Secondly, the liver shows a greater insulin destroying activity than do other tissues (Mirsky and Broh-Kahn, 1949). Weisberg *et al.* (1951) reported that insulin given by the splenic vein in dogs was only about half as effective in lowering the blood glucose as when given by the femoral vein. They estimated the inactivation rate of insulin by the liver of the dog to be 0·02 to 0.04 unit/kg./hour. Therefore to produce the same fall in blood glucose it would be necessary to give a larger dose of insulin intraportally. From these and our own results the following inference may be made. For the same blood glucose lowering effect, the concentration of insulin in the portal blood and the uptake by the liver will be higher than our figures given above. This may be significant when considering the therapy of diabetes mellitus. A diabetic patient is stabilized on subcutaneous insulin to maintain normal blood glucose. Under these conditions it seems probable that the liver is continuously receiving less insulin than a non-diabetic with a similar blood glucose level. The provocative question arises as to whether this could influence the natural history of treated diabetes.

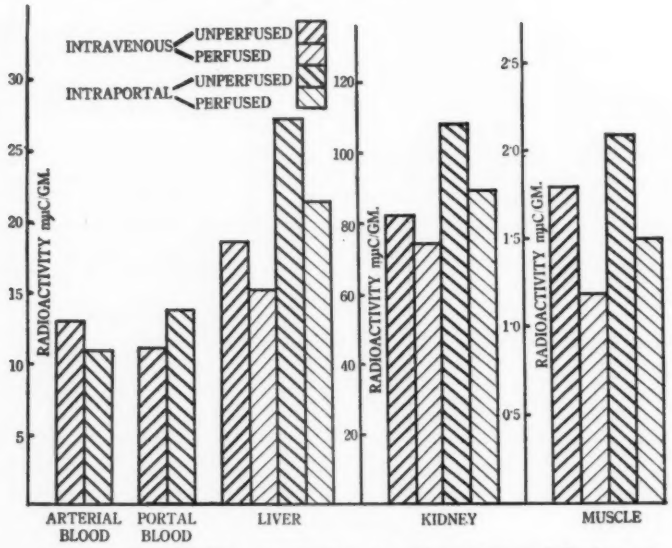

Fig. 5. The PBI^{131} concentration in various tissues following continuous infusion of labelled insulin into the I.V.C. or portal vein (8 rats used in each group). Blood and tissue samples were removed after 1 hour while the infusion was still in progress. The rats were then perfused with warm Ringer's solution and further tissue samples removed.

Binding of PBI^{131} to tissues.

The results comparing PBI^{131} content in the perfused and unperfused tissues are represented graphically in Fig. 5. Most of the PBI^{131} remains in the tissues even after perfusion. The liver and kidney lose only 10-20 p.c. and the muscles 30 p.c. of their PBI^{131} content after perfusion. Actually the loss from the tissues is somewhat less than these figures would indicate because the blood is washed out and there is a tendency for the organs to become a little oedematous during the perfusion.

In similar experiments using I^{131} labelled prolactin it was found that only 0-30 p.c. of the radioactivity in the ovary was lost by perfusion, whereas, after administration of I^{131} labelled bovine serum albumen, 90 p.c. of the radioactivity which localized in the ovary was lost after perfusion (Sonenberg *et al.*, 1951b). If a hormone is retained in an organ after perfusion it may indicate some specific relationship or binding of that hormone to the organ.

Our *in vivo* results with labelled insulin confirm the *in vitro* studies of Stadie (1951) in which he clearly demonstrated that insulin combines or attaches itself to the isolated rat diaphragm. The combined insulin resisted the dissociating action of prolonged washing with saline.

TABLE 2.

Comparing the concentration of PBI^{131} in μC./gm. of tissue 1 hour after a single intravenous injection, or 1 hour of continuous intravenous infusion. 8 rats were used in each group.

	Concentration of PBI^{131} in μC./gm. of tissue	
	Single injection	Continuous infusion
Arterial blood	4·4	12·9
Liver	10·1	18·3
Kidney	30	82
Muscle	1·3	1·8

Single quick injection compared with continuous infusion.

Table 2 shows the blood and tissue contents of PBI^{131} (1) at the end of one hour after a single quick injection into the tail vein, or (2) following one hour of slow continuous infusion into the inferior vena cava.

The concentration of PBI^{131} in the blood and tissues was higher in the rats receiving the continuous infusion. Continuous infusion up to 2 units of insulin per hour approximates closer to physiological conditions than the same dose given as a single quick intravenous injection. This is in accord with the well known observation that a much larger dose of insulin is required to produce the same effect when given as a single quick intravenous injection as compared with subcutaneous administration. In the latter case, the insulin is only slowly

absorbed into the circulation. The results also indicate that if insulin is given intravenously for the treatment of diabetic coma, then it should be given continuously over some hours rather than in a single quick intravenous injection.

SUMMARY.

Crystalline insulin has been labelled by direct iodination with I^{131}. This process does not result in loss of biological activity.

The distribution and disappearance of PBI^{131} has been studied, using intravenous injection and slow continuous systemic or intraportal infusion. The implication of the results has been discussed.

Only a small fraction of the PBI^{131} in muscle, liver and kidney can be removed by perfusing the animal with Ringer's solution.

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